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Engineering a Switchable Nanosystem for Customizable Therapeutics

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ENGINEERING A SWITCHABLE NANOSYSTEM FOR CUSTOMIZABLE THERAPEUTICS

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Abstract

Exosomes are nanovesicles that are naturally secreted by mammalian cells *in vivo* for intercellular communications. Due to their inherent targeting ability, exosomes have a potential for therapeutic applications. However, due to their physiological derivation, the isolation of engineered exosomes has been a major obstacle to their therapeutic application, and successful disease-targeting has been difficult to control. Recently, we have developed an exosome technology that borrows from switchable Chimeric Antigen Receptor (sCAR) T-Cell Therapy and Strep-tag engineering to overcome these obstacles. Here, we describe the development of a *de novo* method to produce genetically modified exosomes with switchable targeting ability and easier isolation capacity. We have elected to fuse an exosome-anchoring protein, vesicular stomatitis viral glycoprotein (VSVG), with a peptide neoepitope (PNE) short-chain variable fragment (scFV) and a StrepTag region. The PNE-scFV will allow for switchable targeting while the Strep-tag will allow for enhanced purification abilities. We have shown that living human cells can produce the engineered exosomes, and we have isolated the engineered exosomes from producer cells. Post isolation, we have characterized these modified exosomes, and we have begun testing the functionality of the PNE scFV and StrepTag regions. This technology will broaden application of exosomes in a therapeutic setting.

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1. Introduction

1.1. Human disease overview

As humans have become better at treating infectious diseases and other foreign pathogens, disease impact has shifted from these conditions to ones that are a result of the human body abnormalities, such as cancers, autoimmune disorders, and genetic diseases. Every year in the United States alone, more than one million individuals are diagnosed with cancer and over 500,000 people die of cancer[1]. So far, scientists have been unable to develop an effective cure for disease such as cancer, and existing treatment options are often ineffective, extremely invasive, damaging to unaffected tissues, and not patient-specific. Every case of these diseases are extremely unique, and treatment plans must be tailored as such. Recent advancements in therapeutics have improved the specificity of treatment options, but this technology is not well standardized. Our project attempts to create an improved system to tailor treatment plans for patients by taking advantage of the human body's natural communication pathways.

1.2. Exosomes as a solution

Exosomes are extracellular vesicles (EVs) that are naturally produced by cells in the body and are believed to be involved in intracellular communication. They are essentially miniaturized versions of the cells that secrete them, sharing an identical phospholipid bilayer as well as specific biomarkers[2]. Additionally, exosomes also have surface proteins that are able to direct them to specific cell types[3]. The open lumen and targeting capabilities of exosomes make them the perfect mechanism for packaging and delivering medicine to specific cells in the body[4]. Therefore, exosomes have been a major focus over the past few years for therapeutic application. However, current disease-targeting exosome technology is limited in how exosomes may target diseased. And, these approaches are also limited due to the ineffectiveness of current isolation techniques.

1.3. Immunotherapy as a targeting solution

One major advancement in cancer treatment that has been developed over the past twenty years has been the use of immunotherapeutics to direct a host's immune system to treat cancer cells over healthy tissue cells. This is to counteract cancer cells' innate ability to evade host immune systems. Immunotherapy modulates immune system interactions to direct immune cells to cancerous tissue over noncancerous tissue. Current immunotherapy treatment options often lack cancer specificity and are not always effective. As such, more creative approaches must be developed to overcome a cancer cell's ability to evade its host's immune system. One idea that has become popularized in the past 10 years is Chimeric Antigen Receptor T cell (CAR-T) therapy. CAR-T therapy imparts cancer targeting abilities on host T cells cancer previously did not exist. This is done by adding a cancer-targeting domain unnatural to most T cells attached to a natural intracellular domain so this enhanced targeting ability triggers natural T cell responses when encountering cancer cells[5]. However, this advancement comes with certain drawbacks such as potential immune system overactivation and producing cells with limited functionality and durability. Switchable CAR-T (sCAR-T) therapy attempts to overcome these obstacles by providing a switchable receptor that can target a variety of targeting molecules[6]. However, many

of the same obstacles still apply to sCAR-T, so more durable advancements for precision therapeutics must be developed.

1.4. Strep-tag as an isolation solution

Biotin-Streavidin binding is one of the most powerful binding affinities found in nature. Strep-tag is an engineered version of biotin that has been applied in many therapeutic settings. We intend on attaching a Strep-tag to our exosomal technology in order to allow for more specific binding. When impure exosome solutions are introduced into a streptavidin column or bead sample, the Strep-tag coated exosomes will be separated from other particles and contaminants that exist in biological samples.

1.5. Applying immunotherapy and isolation techniques to exosomes

The technology which we propose enable specific isolation and tissue-targeting ability by implanting a Strep-tag and switchable targeting system into the surface of exosomes. This allows exosomes to be produced on a large scale without the need for specificity and the ability to be easily purified. Following production and isolation, they can then be "programmed" to target any known cell-surface protein by attaching an antibody specific to that protein to our engineered exosome. To do this, we have chosen to use exosomes to an engineered GCN4 peptide neoepitope (PNE) sequence linked to a cancer marker single chain variable fragment (scFv) should be sufficient[7]. The scFv linked to the GCN4 PNE can be easily swapped out and joined to another scFv, providing our engineered exosomes with an advanced targeting system, capable of targeting any surface protein to specifically deliver appropriate therapeutics. This means that therapeutic exosomes could be designed to preferentially deliver therapies to cancerous or diseased cells, decreasing the non-cancerous tissue targeting and invasiveness of treatment compared to existing therapies. Manipulation of exosomes remains a new prospect in the field of biotechnology, so successful proof of concept could pave the way for an entirely new generation of customizable, precision therapeutics.

1.6. Project goals & outline

For our project, we propose four major goals to discern the applicability of our technology. First, we would want to confirm that our designed protein may be produced by human cells through transient transfection of cells with our engineered construct and the subsequent imaging of producer cells via confocal microscopy. Then, to confirm that our engineered protein is being produced on exosomes, we will co-transfect human cells with known exosomal markers and visualize them to confirm co-localization. Second, we will confirm that our producer cells can successfully secrete our engineered technology through exosomal isolation. We will confirm this isolation through the imaging of isolated exosomes. Third, we will characterize the structure of the exosomes through Western Blot and Nanoparticle Tracking Analysis. Fourth, we will analyze the functionality of the Strep-tag and PNE scFV regions. We will begin by introducing the exosomes into a sample of streptactin coated beads. This will hopefully allow us to isolate our exosomes from other particles in the sample. After successful isolation, the switchable targeting region can be tested by designing a GCN4 PNE with an additional RFP sequence, which will

serve two purposes. Firstly, it will allow us to visualize co-localization between the eGFP sequence on our exosomes and the RFP expressed on our GCN4 PNE so that we are able to visualize co-localization via confocal microscopy. Additionally, the RFP sequence can also be targeted using a Western Blot to further confirm proper protein design.

2. Background and Significance

2.1. Literature Review

2.1.1. Exosome Biogenesis

Exosome biogenesis begins in the nucleus where signaling peptides direct the transcription and translation of proteins that are eventually integrated into the exosomal membrane[4]. Then, proteins are expressed, cellular lipid bilayers invaginate to form early and then late endosomes[4]. As late endosomes are produced, the membrane of the endosome invaginates again to form a multivesicular body (MVB)[4]. Afterwards, the MVB will go through one of two pathways. The first MVB pathway is in which it will combine with internal lysosomes that will begin breaking down the MVB to recycle its components[4]. In the other pathway, the MVB will fuse with the cellular plasma membrane and release exosomes[4].

2.1.2. Exosomes as Biomarkers and Therapeutic Delivery Devices

Cell utilization of exosomes is not yet completely understood, but some believe that the primary function of exosomes is intercellular communication[4]. One aspect of exosomes that is currently being explored is the implications of exosomal intraluminal biomarkers as indicators of cellular health. Exosomal biomarkers typically consist of proteins, DNA, and RNA that are produced by the progenitor cell[2]. These biomarkers can indicate diseases such as diabetes, cancer, and neurodegenerative conditions such as Alzheirmers[2]. Another area of research is the innate extraluminal cellular-targeting mechanism of exosomes. When exosomes are applied for therapeutic purposes, this targeting system can be engineered to preferentially target any surface protein on another cell[4]. After exosomes are engineered to target specific cell types, passive or active upload can be used to pack therapeutic components into exosomes. In passive loading, a concentration gradient or drugging of donor cells is used to upload small molecule drugs into exosomes[4]. In active loading, techniques such as sonication, extrusion, freeze/thaw cycles, electroporation, incubation with saponin, click chemistry, and antibody binding are used to load therapeutic cargo into exosomes[4].

2.1.3. Conventional CAR-T and Switchable CAR-T (sCAR-T)

As cancer cells mutate, they develop an innate ability to evade their host's immune system to ensure their own survival. As such, more creative approaches must be developed to overcome a cancer cell's ability to evade its host's immune system. One idea that has become popularized in the past 10 years is Chimeric Antigen Receptor T cell (CAR-T) therapy. In CAR-T therapy, host T cells are isolated and transduced with engineered lentiviral vectors and DNA constructs[5]. These constructs give cells the abilities to express CARs on their surface. CARs are single chain antibody variable fragments (scFV) fused to intracellular signaling domains[5]. This gives T cells the targeting abilities of antibodies while still holding onto their appropriate intracellular cascade[5]. Traditional CAR-T is limiting in that its targeting abilities cannot be altered after

transduction which can cause adverse side effects for patients and can limit CAR-Ts overall functionality[6]. Switchable CAR-T (sCAR-T) improves upon the drawbacks of traditional CAR-T by replacing the targeting scFV with a switchable domain where any targeting scFV can be added[6]. This allows CARs to be "shut off" or change their targeted protein by replacing the protein bound to the switchable domain of the CAR[6].

2.1.4. Antibody-Antigen Binding

Antibodies (Abs) are proteins produced and secreted by B-cells as part of an organism's adaptive immune response. Each antibody is composed of two light chains fused to two longer heavy chains through disulfide bonds. Additionally, each antibody can be separated into two distinct regions, a variable Fv (also known as Fab) region, which interacts with antigens (Ags), and an Fc effector region, which allows the Ab to assist other immune cells in neutralizing the foreign threat.

Within the Fv region, the extremely diverse Ag-binding region is of particular interest since Abs have the capability to interact with upwards of 10^{11} different antigens in humans. This variability is primarily imparted by three subregions known as complementarity-determining regions (CDRs)[7]. CDRs on both light and heavy chains play a role in determining what specific Ag an Ab is capable of binding to. If the exact protein sequence of an Fv can be determined, this can be extracted and synthesized onto other proteins, thereby granting them the ability to target a specific Ag as well.

2.1.5. Peptide Neo-epitopes

Peptide neoepitopes (PNEs) are mutated short peptide chains that contain slight amino acid variations from their original epitopes. They are commonly produced by cancer cells and can be detected by the immune system, which can then initiate an immune response and result in tumor rejection[8]. PNEs of particular interest produced by cancer cells are immunogenic epitopes known as neoantigens, because they can be studied for therapeutic purposes[8]. Successfully classifying neoantigens provides the ability to offer personalized treatments for cancer patients and the technology can also be applied for diagnostic purposes as well.

2.1.6. Streptavidin Binding

The binding of the streptavidin (SA) bacterial protein to a small molecule of biotin is one of the strongest naturally occurring binding interactions. This high-affinity binding is an extremely powerful biological tool that is exploitable in biology to study high-affinity protein-ligand binding[9]. Despite the unnaturally strong binding affinity, the interaction is still prone to failure under certain biological conditions, such as low pH, high temperatures, or when being attached to endosomes[9].

2.2. Drawbacks of Current Technologies

2.2.1. sCAR-T

Traditional CAR-T therapy has many advantages as it gives T cells tumor-targeting and signaling abilities that allow it to overcome cancer's unique ability to evade host immune systems. However, this comes at a cost. T cells become so efficient at killing with their new

abilities that they are difficult to regulate. They have been shown to target non-tumor cells with similar characteristics to their cancerous counterparts[10]. This extremely efficient killing process has not only led to healthy tissue targeting but also to more extreme conditions that can sometimes be fatal. One of these conditions is Cytokine Release Syndrome (CRS) and Tumor Lysis Syndrome (TLS)[11] . These two are caused by unregulated immune system activation which leads to robust cell death and organ malfunction. CRS is the result of engineered cells being overstimulated and over-releasing cytokines upon reintroduction to the body. TLS is caused by the over-efficient killing of tumor cells by engineered T cells. sCAR-T cells improve upon these issues as they may be manipulated or deactivated *in vivo*, but they do not fully address the issue.

Another major drawback of current CAR-T and sCAR-T technology is their reliance on currently available isolation and transduction procedures. T cells are isolated from blood by an invasive process called leukapheresis. This process places a central line into a patient where blood is removed, and T cells are isolated. Then, the process of transducing T cells may take 2-6 weeks to accomplish[2].

2.2.2. Exosomes

While exosomes for therapeutic purposes is one of the fastest-growing fields within biotechnology, there are still many challenges researchers face. A primary drawback of exosome technology is the immense difficulty encountered when attempting to isolate exosomes. Even isolating exosomes *in vitro* in an extremely controlled environment is difficult because cells secrete heterogenous EVs, most of which share a size overlap with exosomes. As such, attempts to isolate exosome samples often still result in impure samples with co-isolated other EVs[2]. Various isolation methods have been developed, with limited success, and greatly affected exosome yield.

Another common issue with exosome research is characterization of nanoparticles. The challenge of characterizing exosomes is compounded by the heterogeneity of EVs that were isolated alongside exosomes. Oftentimes, analysis will return mixed size distributions, making it near impossible to properly categorize the exosome sample[12]. Again, many solutions have been proposed with varying degrees of success, such as direct labeling of exosomes with a fluorescent protein that is located in the exosomal membrane. However, given the nanoscale of EVs, most categorization techniques remain far from perfect.

2.3. Project Goals and Constraints

2.3.1. Protein design and production

As stated above, our project uses natural cell mechanisms to produce our technology. Therefore, our first step was to establish multiple cell lines with proper mammalian cell culture passaging principles. Next, we needed to design a DNA plasmid construct that would produce our engineered exosomes with desired surface proteins. To confirm proper protein design, we added an enhanced Green Fluorescent Protein (eGFP) on the inner domain of our exosome so that we could visualize the protein as it was produced by cells and loaded onto exosomes. This fluorescent marker could be replaced in the future by genetic material or protein-based therapeutic. We introduced our designed DNA into cells via transient transfection with polyethylenimine (PEI). And, after confirmation that the desired protein was being produced

with fluorescent and confocal microscopy, we performed a cotransfection with our designed construct and other constructs with known exosomal markers. If designed correctly, these signals should overlap.

2.3.2. Exosome isolation and purification

After proper protein design and production was confirmed, we isolated exosomes from their host cells using centrifugation and with other commercially available reagents. We confirmed that the isolation was successful by visualizing via confocal microscopy. To improve upon current isolation procedures, we introduced a novel Strep-tag onto our surface exosome that may be used to isolate our particles from others of the same size. We used Stepavidin-tagged agarose beads to bind our exosomes and further isolate them from other particles.

2.3.3. Exosome and protein categorization

Next, we had to categorize our exosomes to ensure they retained normal exosome functionality as well as the functionality imparted by our designed protein. In order to do this, we first performed exosome uptakes *in vivo*, by introducing our exosome samples to multiple cell lines and visualized their uptake (or lack thereof) into plated cells over a 72 hour period using confocal microscopy. Additionally, we also sent a sub-sample of our transfected exosomes out for nanoparticle tracking analysis (NTA) to compare the size of our exosomes to their natively-produced counterpart, providing substantial quantitative data to supplement our largely qualitative data. We also performed a Western Blot binding assay to semi-quantitatively characterize the size of our designed protein.

2.3.4. Protein functional analysis

Finally, to truly confirm our exosome technology functions as expected, we had to test the scFv binding site with a peptide neo-epitope (PNE), or a slightly modified version of GCN4. This was done by designing another DNA plasmid containing copies of the GCN4 PNE (amino acid sequence: NYHLENEVARLKKL), Gaussia Luciferase (GLuc) and red fluorescent protein (RFP) reporter genes, along with an Interleukin-2 (IL-2) signal sequence. This plasmid was co-transfected with our original DNA plasmid *in vivo* and the interaction of the secreted products was monitored for co-expression. Additionally, the GLuc allowed us to perform a luciferase assay on our samples to obtain quantitative data alongside.

2.4. Significance

If successful, this project has a multitude of ramifications across the therapeutic space. Until now, small-molecule drugs have many limitations in terms of their specificity and longevity within the body. The uploading of therapeutics into labeled nanovesicles has the potential to overcome these barriers by increasing the half-life of therapeutics and increase specificity by applying biomarkers to the surface of nanotechnologies. However, non-native nanotechnologies may trigger an immune response, and they can often be difficult to work with. Human cell-derived nanovesicles, termed exosomes, may function better than other nanotechnologies due to their innate ability to more easily interact with the body *in vivo*. But, exosomes are still limited due to their narrow applicability and inability to be easily produced on a large scale. We have applied recent advancements in immunotherapy to overcome these areas by providing a simple

exosome template that can be easily adapted to different applications. Specifically, we embedded a sCAR-T-like protein into the surface of our exosomes so that the surface protein could be switched depending on the desired target for the exosomes. This technology has the ability to target a multitude of cell types throughout the body and deliver a variety of therapeutic cargo *in vivo* delivery. This system can be applied to overcome obstacles presented by conditions such as cancer, genetic diseases, and inflammatory diseases. And, visualizable markers can be placed on the surface of exosomes for *in vivo* imaging of exosome progression.

3. Systems Level Analysis

3.1. Exosome Protein

The exosome protein is to be embedded into the lipid bilayer of exosomes. The protein will be introduced to cells via transfection, and because of the inclusion of certain elements of our protein, it will allow the cell to directly produce the desired technology by targeting exosomal precursors. A schematic of the entire protein design is shown below in **Figure 1**.

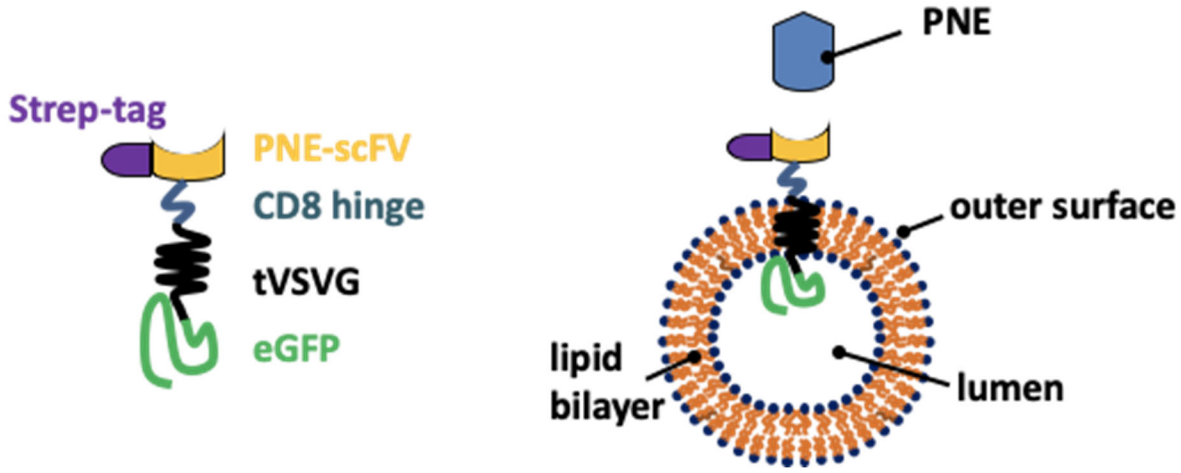

Figure 1. Schematic of exosome protein and interaction with target PNE after successful transfection.

3.1.1. Strep-Tag II

Strep-Tag II (highlighted in purple in **Figure 1** and referred to as Strep-tag) is a small, engineered molecule designed to mimic the biotin molecule (**Section 2.1.6**)[13]. Instead of binding to the streptavidin, Strep-tag is designed to bind to a similarly engineered version of streptavidin known as Strep-Tactin[13]. This interaction has a slightly weaker affinity than the naturally occurring biotin-streptavidin, although it is still extremely strong. Strep-tag was included in the protein design to alleviate the issues related to exosome purification (**Section 2.2.2**). After obtaining the successfully transfected exosome system, the Strep-tag present on the external surface of the exosome will be able to interact with Strep-Tactin that has been immobilized to the surface of a resin-bead. By introducing both of these into a heterogeneous solution and mixing them together for a period of time, exosomes successfully expressing a Strep-tag will

bind to the Strep-Tactin coated beads, allowing us to take advantage of this binding to separate bound exosomes from other nanoparticles present in the solution.

3.1.2. Peptide Neo-epitope Single Chain Variable Fragment (PNE-scFV)

PNE-scFV is the critical design segment of or exosome protein (highlighted in yellow in **Figure 1**). Incorporating the PNE-scFV segment into our protein design allows for a one-design, multiple-target approach. Borrowing the same ideas from sCAR-T therapy (**Section 2.1.3**), the exosome protein first binds to an intermediary PNE, which is in turn bound to another scFV, such as anti-CD19, which specifically targets the CD19 surface marker found on cancer cells within the body[14]. If another surface marker is to be targeted, such as Her2, instead of having to re-engineer the exosome system from scratch, only the scFV that is bound to the PNE needs to be modified to anti-Her2 (instead of anti-CD19), whilst the exosome system still binds to the PNE. The PNE that was chosen to be targeted is GCN4, and just like the scFVs bound to the PNE, the PNE-scFV segment itself is anti-GCN4, allowing the exosome system to bind to the GCN4 PNE[14].

GCN4 was chosen as the target because it is also commonly used in sCAR-T therapy. GCN4 is an ideal target because it is a yeast transcription factor, meaning it is not naturally occurring within the human body, allowing human antibodies, in this case, the PNE-scFV, to target it[6]. Despite being a foreign epitope sequence, extensive studies have shown it has little to no immunogenicity, further making it the ideal target since the binding of PNE-scFV to GCN4 will cause no adverse side effects[6].

3.1.3. CD8 Hinge

A CD8 hinge region (highlighted in blue in **Figure 1**) was incorporated into our protein design to increase the flexibility of the external surface segment of our protein, and allow Strep-tag and PNE-scFV to move more freely and better bind to their intended targets. The CD8 hinge is a commonly used structure in other immunotherapy approaches, such as that of CAR and sCAR-T Therapy[14].

3.1.4. Truncated Vesicular Stomatitis Virus Glycoprotein (tVSVG)

tVSVG (highlighted in black in **Figure 1**) is, as the name suggests, a slightly modified, shorter version of the glycoprotein segment of a vesicular stomatitis virus. The inclusion of this gene segment in our exosome protein design is twofold. Firstly, tVSVG increases the transfection efficiency of the naked plasmid vector, which is desirable since our protein is being introduced via transfection. Secondly, tVSVG has been specifically modified to be a transmembrane protein that specifically targets exosomal precursors within cells, allowing transfected cells to directly produce the engineered exosome technology[15]. Additionally, since tVSVG is transmembrane in nature, it allows us to connect external and internal surface proteins onto the same protein.

3.1.5. Enhanced Green Fluorescent Protein (eGFP)

Lastly, eGFP (highlighted in green in **Figure 1**) was included in our protein design for visualization purposes. eGFP expresses a slightly more robust signal than traditional GFP, and combined with our

exosomal isolation process, allows us to visualize the nanoscale exosomes on the microscale using confocal microscopy[16]. Additionally, since the eGFP signal is so robust, it also produces an easily distinguishable signal, which is also important because the autofluorescence of non-transfected nanoparticles is a concern at this scale[16].

4. Methods and Materials

This is a list outlining the methods and materials that have been used throughout our project. Mammalian cell culture protocols were used throughout the project to maintain various cell lines. Transient transfection was used to introduce our designed plasmid into the cultured cell lines so that cells would begin producing our desired exosomal protein construct. Cell microscopy was used to confirm the presence of our designed protein both inside of the cell and outside of the cell on exosomes. Hoechst nuclear stain was used to confirm that cells' nuclei were intact during cell microscopy.

Exosome isolation was done to separate exosomes from the extracellular matrix after they are produced and secreted by cells. Exosome imaging was done to confirm that isolated exosomes did express the correct protein on its surface. Exosome uptakes were performed to confirm that our engineered exosomes retained native exosome function. Exosome staining was done to supplement the natural signal produced by our engineered exosomes. These stained exosomes were then introduced to Streptavidin-tagged beads to confirm the functionality of one portion of our protein design. The Streptavidin-tagged beads may be used to further purify and isolate exosome samples in the future.

Nanoparticle Tracking Analysis was done to compare the size of our exosomes to their natively-produced counterpart, providing substantial quantitative data to supplement our largely qualitative data. DNA Maxiprepartation was done to multiply our designed DNA for when our existing stock is depleted, providing a cheaper alternative to purchasing more directly. Western Blot is used to semi-quantitatively characterize the size of our designed protein. Together, these experiments have allowed us to confirm the successful transfection of our engineered exosomes, along with categorizing their structure and function.

4.1. Mammalian Cell Culture

4.1.1. Cell Passaging

Human kidney cells (293T) (Alstem; Richmond, CA, USA) and Human hepatocellular carcinoma cells (Hep-G2) (American Type Culture Collection; Manassas, VA, USA) were cultured at 37ºC and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco; MA, USA), which contained 10% Fetal Bovine Serum (FBS) (GE Healthcare Life Sciences; Issaquah, WA, USA), and 1% Penicillin Streptomycin (Pen Strep) (Gibco; MA, USA). These cells were passaged at 70-80% confluency. Cells were first washed with Phosphate Buffered Saline (PBS) (Teknova; Hollister, CA, USA) preheated to 37ºC to remove cell debris. Then, monolayer cells were detached from the culture plate with 0.25% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) (Gibco; MA, USA) by incubating for 5 minutes at 37ºC. Following incubation, the trypsin-EDTA was deactivated with DMEM media, and the suspended cells solution was centrifuged at 1500 rpm for five minutes to separate the cell pellet. After resuspension in DMEM, the cells were reseeded on a new plate. For general passaging, 60 mm plates were used. For

transient transfection (**Section 1.2**), four-chamber 35 mm plates were used. For exosome isolation (**Section 2.1**), 145 mm plates were used.

4.1.2. Transient Transfection

Transfected plates were confirmed to be at 50% confluency for imaging plates and 70-80% confluency for other plates via cell microscopy (**Section 1.3**). If using a 35 mm imaging plate, cells were transfected with a solution consisting of 200 µL Opti-Mem (Gibco; MA, USA), 10 µL polyethylenimine (PEI) transfection reagent, and 2 µg of DNA plasmid. Afterwards, cells were incubated for 24 hours at 37ºC before imaging via cell microscopy (**Section 1.3**) to confirm successful transfection. If transfecting with multiple plasmids (i.e. for a co-transfection), the same procedure was with the exception of the total DNA weight being divided between the total amount of plasmids. For example, 1 ug of each plasmid was used in the case of two plasmid types.

4.1.3. Cell Microscopy

Cells were imaged using either fluorescence (Olympus; Waltham, MA, USA) or confocal microscopy (Leica; Buffalo Grove, IL, USA). Software used for image capturing was either cellSens Standard (Olympus; Waltham, MA, USA) or Leica Application Suite X (Leica; Buffalo Grove, IL, USA).

4.1.4. Hoechst Nuclear Staining

A stock solution of 1 mg/mL Hoechst 33352 (ThermoFisher Scientific; Fremont, CA, USA) was further diluted to a working solution at a ratio of 1:1000 Hoechst to PBS was created. After cells were washed with PBS, the working solution was added to the cells and incubated for 10 minutes at 37ºC. Cells were then washed with PBS and visualized using confocal microscopy (**Section 1.3**).

4.2. Exosome Isolation and Characterization

4.2.1. Exosome Isolation

293T cells were cultured on 145 mm tissue culture dishes (**Section 1.1**) until reaching 60-70% confluency. The 293T cells were then transfected using a scaled-up transfection solution (2 mL OptiMEM, 100 µL, and 20 µg DNA plasmid) (**Section 1.3**). After an additional 24 hours or until reaching 80-90% confluency, transfection efficiency was confirmed via fluorescence microscopy (**Section 1.3**), and cell media was replaced with serum-free UltraCulture (Lonza; Portsmouth, NH, USA). After incubating in UltraCulture for 48-72 hours, the conditioned media was collected and centrifuged for 1500g for 10 minutes and steadily filtered through a 2 µm filter. Then, ExoQuick-TC (System Biosciences; Palo Alto, CA, USA) exosome isolation reagent was added to the UltraCulture media to clump exosomes together. After incubation at 4ºC overnight, conditioned media was centrifuged at 3000g for 1.5 hours. The supernatant was removed and isolated exosomes were resuspended in PBS. Finally, the protein concentration was then measured using a NanoDrop Lite (Thermo Fisher Scientific; Fremont, CA, USA).

4.2.2. Exosome Imaging

2-3 µL of the isolated exosome sample at approximately 1 µg/µL (**Section 2.1**) was plated on a 35 mm four-chamber glass bottom tissue culture plate. The plate was then incubated for 5 minutes at 37ºC to allow the exosome reach the bottom of the plate. Following incubation, the exosomes were imaged via confocal microscopy (**Section 1.3**).

4.2.3. Exosome Uptake

293T or HEPG2 were passaged (**Section 1.1**) and plated onto a 35 mm four-chamber glass bottom tissue culture plate. Upon reaching 40-50% confluency, 25-30µg of transfected exosomes were added to the appropriate wells. Exosome uptake was imaged at 24, 48, and 72 hour time points via confocal microscopy (**Section 1.3**). After 72 hours, Hoechst 33352 was applied to cells (**Section 1.4**), and cells were again imaged with confocal microscopy.

4.2.4. Exosome Staining

300 µL of incubation buffer and 60 µg of isolated exosomes (**Section 1.2**) were mixed in a microcentrifuge tube. Afterwards, roughly 5 µL of 100 µM ExoGlow (System Biosciences, Palo Alto, CA) was added to the tube to bring the final concentration of ExoGlow to 1.5 µM and then wrapped in aluminum foil. Tubes were placed in a 37ºC incubator for 60 minutes, after which Exoquick-TC was introduced (**Section 2.1**) overnight at 4ºC. The following day, the exosome suspension was spun down at 3000g for 90 minutes and then resuspended in PBS ready for use (**Section 2.1**).

4.2.5. Streptavidin Beads

Pierce™ Streptavidin Plus UltraLink™ Resin Beads (Thermo Fisher Scientific; Fremont, CA, USA) were used via manufacturer's protocol. As per this protocol, the Streptavidin-tagged bead solution was thoroughly mixed, and then 10 µL of beads were added into a microcentrifuge tube. The bead solution was then spun down at 4000g for 1 min, before aspirating supernatant and resuspending with PBS. This wash step was repeated once before introducing the stained exosome sample (**Section 2.4**) and incubating at room temperature on a rocker for 60 minutes. After the hour wait step, the solution was again spun down at 4000g for 1 min, the supernatant removed, and resuspended in PBS. This repeated three additional times before fused exosome-bead construct was ready for imaging.

4.2.6. Nanoparticle Tracking Analysis (NTA)

Isolated Exosomes (**Section 2.1**) were suspended in 100 µL of PBS at ~1.3 µg/µL. Control and transient transfection-derived exosomes were sent to Particle Characterization Laboratories Inc. (Novato, CA, USA) for NTA analysis per the company’s protocol.

4.3. DNA Maxiprep

DNA Maxiprep is used to magnify an existing DNA plasmid when running low on the original sample. The DNA plasmid is introduced into E. Coli to be multiplied, after which it will harvested and purified

for additional use in experiments outlined in **Parts 1** and **2**. This will be performed closer to the end of Winter 2020 when our DNA plasmid runs low.

4.4. Western Blot Analysis

Western Blot is a common industry technique used to semi-quantitatively determine the size of proteins within a sample. This experiment will be performed towards the end of Winter 2020 when we have gathered an ample amount of exosome samples to test, since multiple samples can be tested simultaneously. An additional Western Blot may be performed at the start of Spring 2020 to confirm experimental results.

5. Results

5.1. Confirmation of proper plasmid design

The initial stage of our experiment consisted of a series of transfections and co-transfections to confirm proper plasmid design. Since the exosome protein has an eGFP gene segment, successful transfection would result in the expression of eGFP within the transfected cells (**Figure 2**).

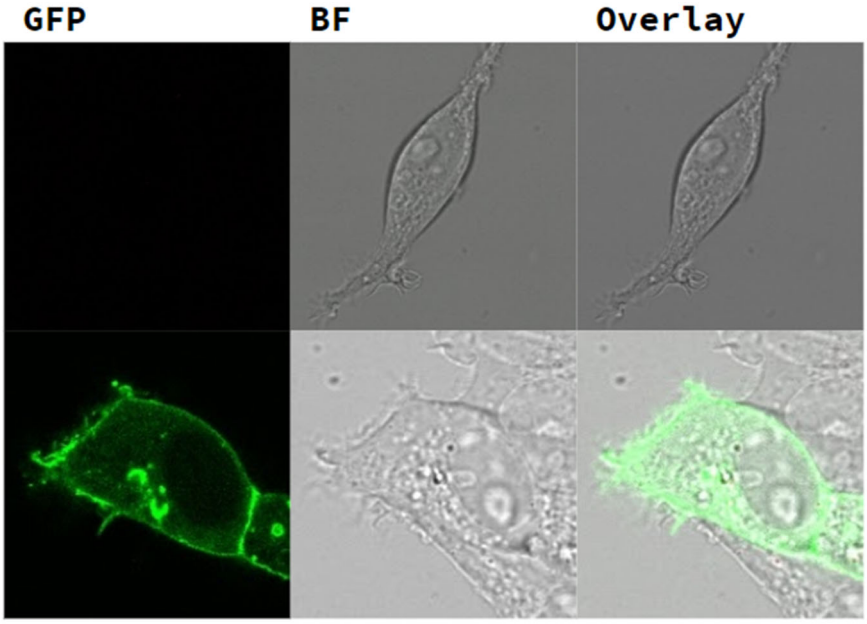

Figure 2. Comparison between control (top row) and transfected (bottom row) 293T cells. The comparison shows a successful transfection of our engineered protein, PNE-eGFP. The first picture in each set is GFP, followed by bright field (BF), and finally an overlay of GFP + BF.

Transfection of a large quantity of foreign DNA is known to be relatively cytotoxic. Therefore, upon observing successful transfection in 293T cells, the next important step was to redo the transient transfection, this time implementing a Hoechst nuclear stain on the last day of the process (72 hours), to ensure the nucleus was still intact, which meant that the transfected cells were still healthy and producing the desired exosome technology (**Figure 3**).

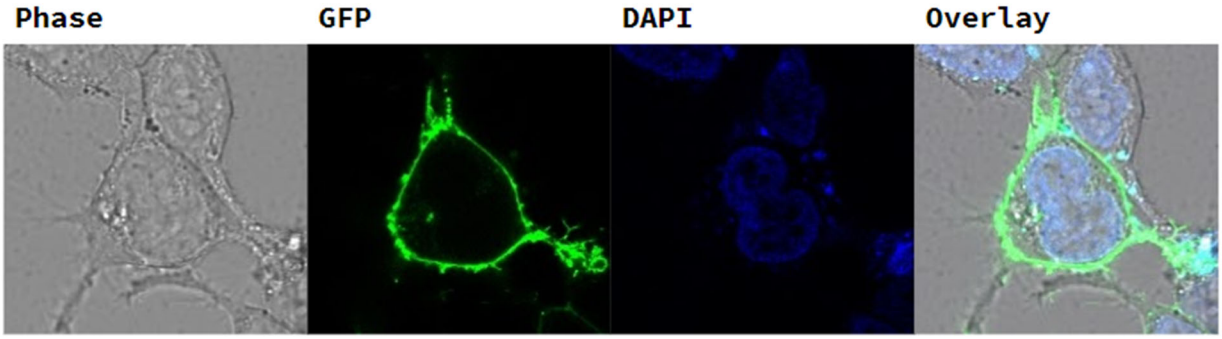

Figure 3. Transient transfection of exosome protein in 293T cells, accompanied with a Hoechst nuclear stain (DAPI). The Hoechst stain was applied on the final day of the transfection process (72 hrs), revealing an intact nucleus, and confirms our transfected cells were able to successfully uptake the foreign DNA plasmid without dying.

However, it is not enough to simply observe successful transfection within the cell, but rather exosomes in particular. By taking advantage of the natural exosome biogenesis pathway, specific exosomal precursors can be targeted and marked with another fluorescent protein. Additionally, exosomes themselves can also be targeted within the cell, right before secretion, and marked with a fluorescent protein in a similar manner. By conducting these transfections in tandem, known as a co-transfection, observing co-localization of GFP and RFP signals is strongly indicative of proper protein plasmid design (**Figure 4**).

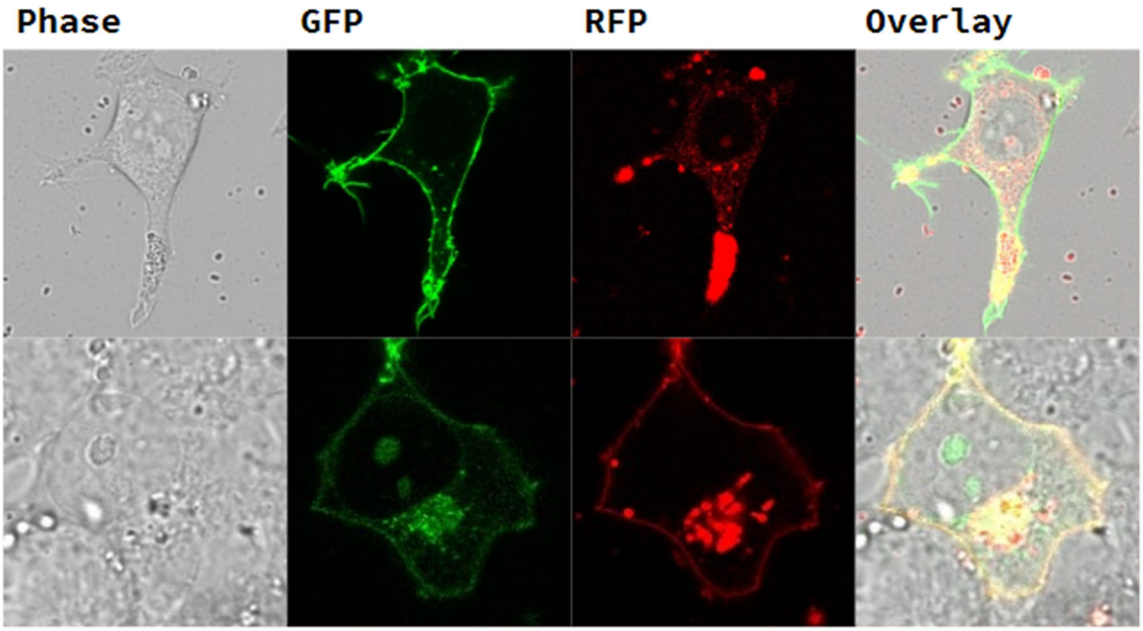

Figure 4. Co-transfection of exosome protein in 293T cells along with: a known late endosome marker (top row), and CD63-RFP, a known exosome marker (bottom row). Both exosomal markers expressed RFP, whereas the exosome protein continued to express GFP. Varying degrees of co-localization of signal are observed between the late endosome and CD63 markers, because late endosomes are an exosomal precursor, whereas CD63 directly targets exosomes.

5.2. Isolation of engineered exosomes

Upon confirmation of proper plasmid design, the next step is to isolate exosomes from other naturally secreted nanoparticles (**Section 4.2.1**). After a series of tabletop centrifugation steps, exosomes can be successfully isolated from other particles present in extracellular fluid only if they differ drastically in size. It is not possible to separate exosomes from similarly sized nanoparticles, which is why the sample is referred to as isolated exosomes rather than purified exosomes. The introduction of Exo-TC before the Harvest II step of exosome isolation causes exosomes to form clumps, which is why they are able to be visualized using confocal microscopy, despite being nanoscale in nature (**Figure 5**).

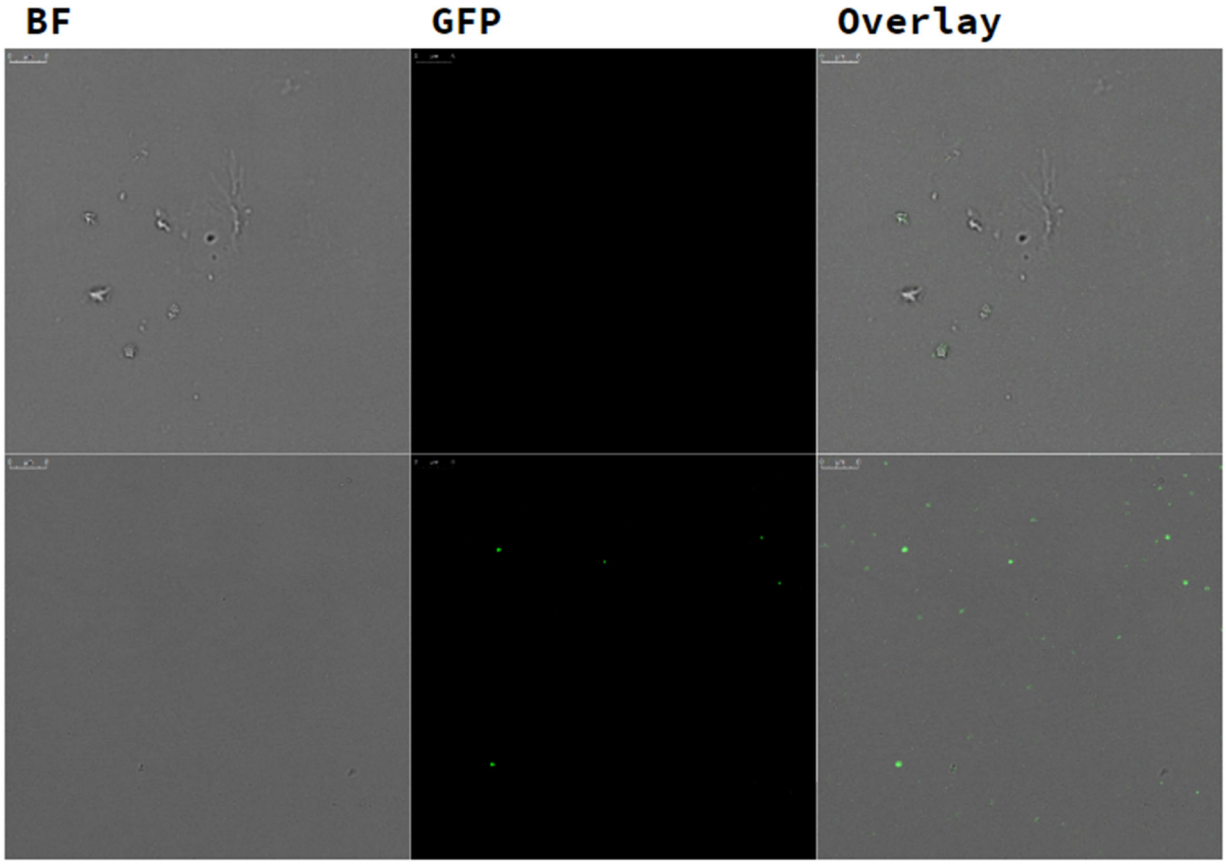

Figure 5. Successfully isolated control (top row) and engineered (bottom row) exosome samples. The extremely faint green present in the control overlay can be attributed to autofluorescence, supported by the lack of any distinguishable signal in the control GFP image. The isolated samples are not purely composed of exosomes, as other similarly sized nanoparticles are present in solution, although a definitive majority of the sample consists of exosomes.

Whilst isolated exosomes are able to be visualized using confocal microscopy, the signal is still relatively weak given how small an individual exosome is. Additionally, due to autofluorescence in both control and exosome samples, part of the signal being visualized may not be due to engineered exosomes at all. As such, it is important to chemically stain the exosomes using an RNA probe to amplify the signal. The

RNA probe specifically targets RNA fragments present in exosomes but not that of other nanoparticles, acting as a secondary check to confirm the presence of exosomes in the isolated sample (**Figure 6**).

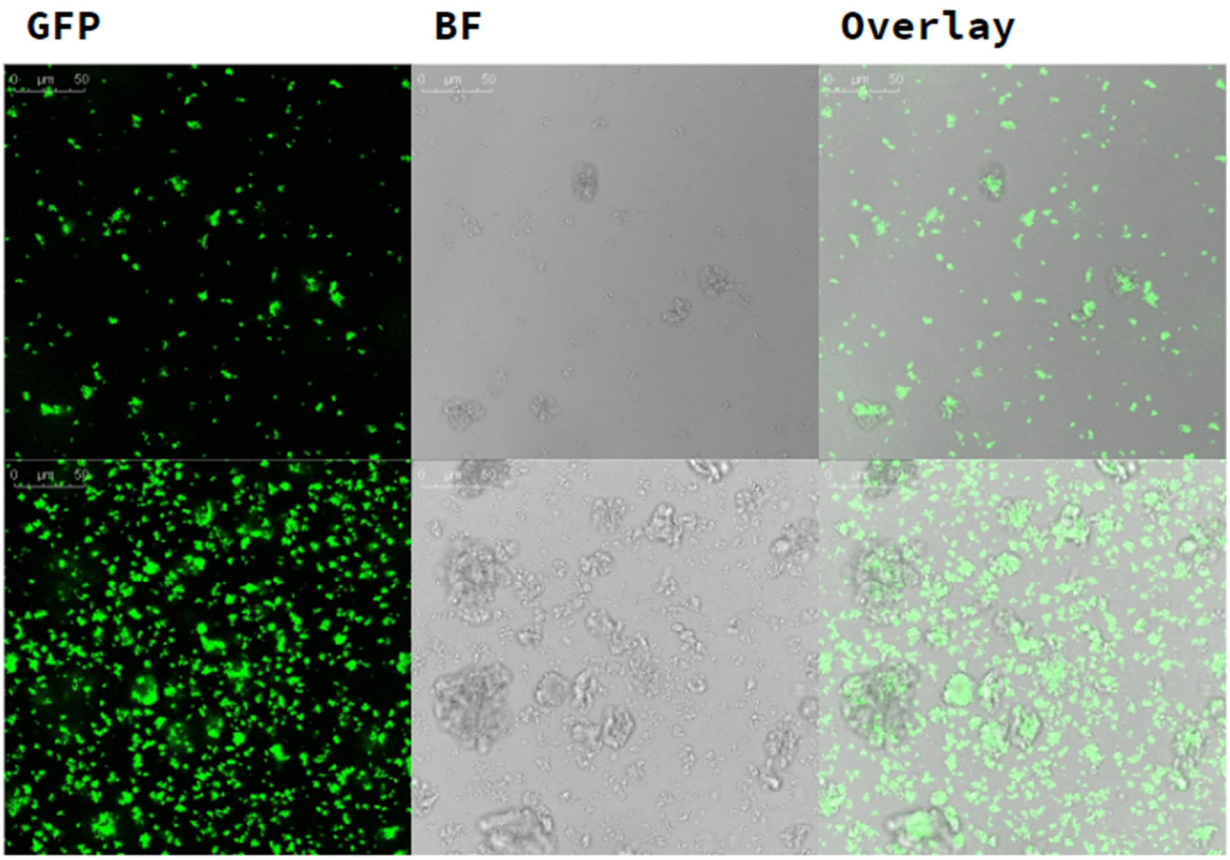

Figure 6. Control (top row) and engineered (bottom row) exosomes chemically stained with an RNA probe. Both samples express an extremely vibrant stain due to the signal amplification caused by the RNA probe.

5.3. Characterization of exosome size and structure

5.3.1. Nanoparticle tracking analysis (NTA)

After successfully isolating an ample amount of exosome samples from both control and transfected 293T cells, we needed to determine if the transfection process had an effect on the structural integrity of the exosomes. To accomplish this, both control and exosome samples were sent out for an NTA, which compares the size of our exosome samples. Size is a key indicator of structural integrity, and then engineered exosomes should remain similar in size, since the transfected protein itself is significantly smaller than even exosomes (**Figure 7**).

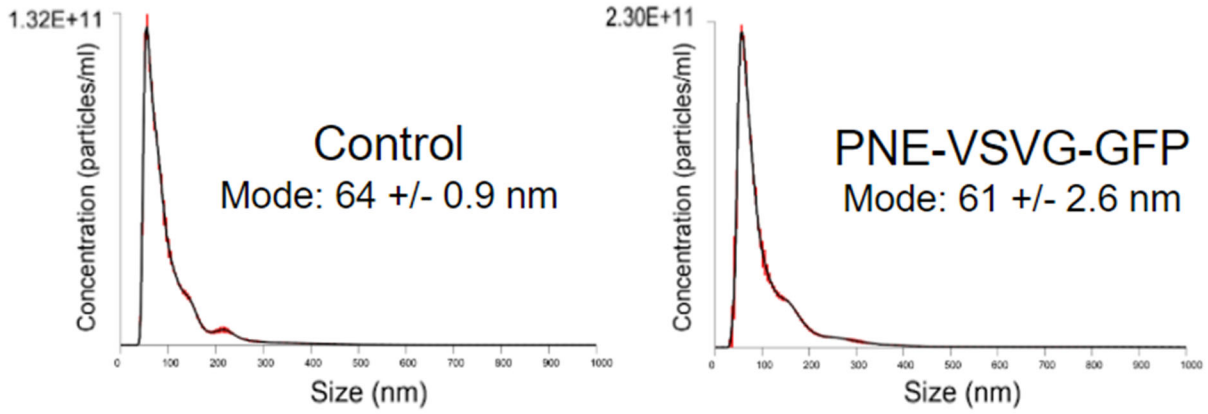

Figure 7. NTA analysis of control and engineered exosome. The analysis of the two samples shows that engineered exosomes remain similar in size to control exosomes, supporting the claim that structural integrity was upheld throughout the transfection process, and that the engineered exosomes managed to retain basic exosome function, in addition to the new targeting abilities imparted through transfection.

5.3.2. Western blot

After obtaining the NTA results, a series of Western blot experiments were performed on both control and engineered exosome samples to semi-quantitatively measure the size of the embedded exosome protein, which was expected to be 70-80 kD in size (**Figure 8**). The antibody target for the Western blot was the eGFP segment of the exosome protein.

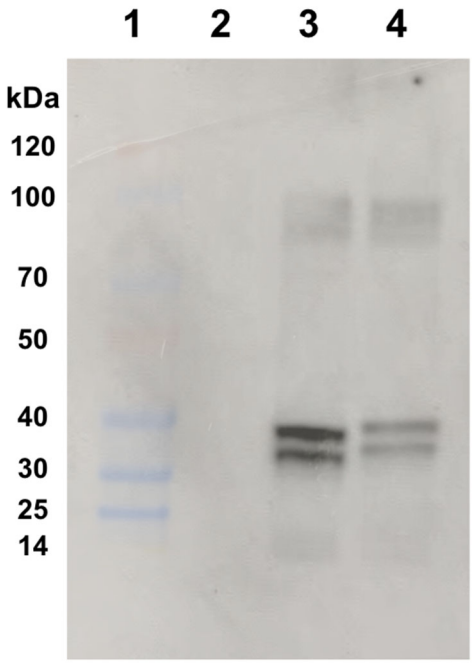

Figure 8. Western blot analysis of one control sample (lane 2), and two engineered exosome samples (lanes 3 & 4). As intended, the control exosome lane is empty because there was no exosome protein containing eGFP present. However, both engineered exosome samples returned very similar results, with a smeared band at roughly 70-80 kD, and two distinct bands at 30 and 40 kD, which can be attributed to protein shearing during the Western blot process.

5.4. Functional analysis of key protein components

5.4.1. Purification using Strep-tag II

Upon completing the classification stage of the experiment, the final step is to ensure the functionality of key components of the exosome protein design. The first key component is Strep-tag, which was used to successfully isolate transfected exosomes from other nanoparticles found in the exosome solution that did not express Strep-tag. The engineered exosome sample was mixed in solution with Strep-Tactin coated resin beads for a set time, after which the beads were then removed, in the process purifying the bound exosomes (**Figure 9**).

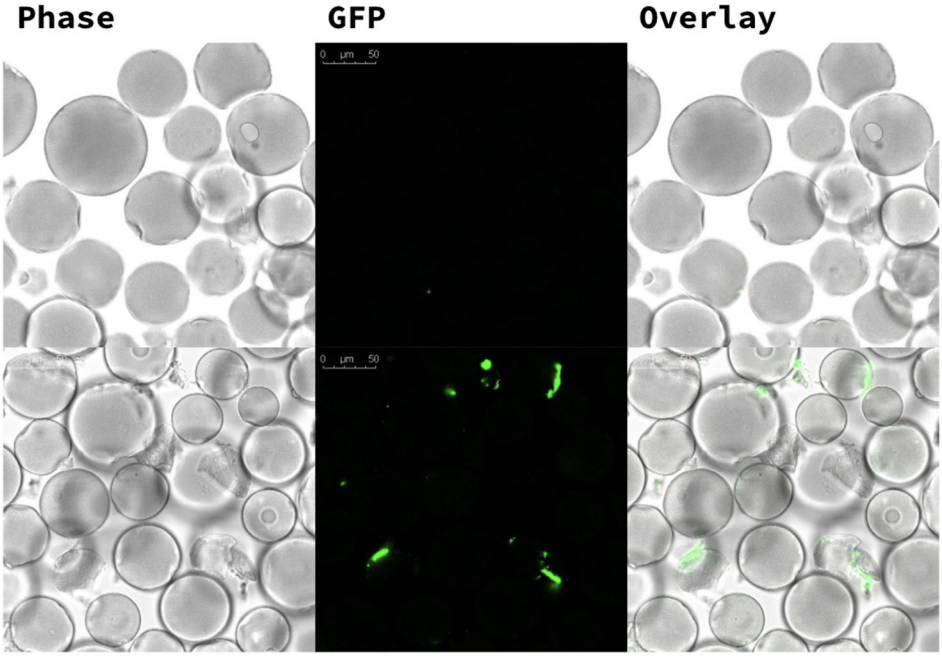

Figure 9. Purification of engineered exosomes (bottom row) utilizing the strong Strep-tag/Strep-Tactin binding affinity. Only exosomes that expressed Strep-tag on their surface were able to bind to the Strep-Tactin coated resin beads, whereas those that did not were left in solution. Other nanoparticles were also unable to express Strep-Tactin, thus also unable to bind to the beads.

5.4.2. Interaction of PNE-scFV with GCN4 PNE

Due to the COVID-19 pandemic, this final stage of the project was unable to be completed (**Section 7.2**). An additional protein, containing *Gaussia* luciferase (GLuc), ruby RFP (rRFP), and GCN4 genes was designed to be co-transfected in 293T cells. Due to the inclusion of rRFP, successful binding between GCN4 and PNE-scFV would result in being able to visualize co-localization, similar to that of **Figure 4**.

6. Engineering Standards and Realistic Constraints

6.1. Health and Safety

Health and safety was of the highest priority when designing our technology. Our biggest focus here was first and foremost the safety of the patients that were being helped by our proposed technology. First, we made sure that the extravesicular region of our technology is Because exosomes are human-derived, the surface proteins engineered exosomes may have protein markers on their surface that were not intended on being there. Therefore, further preclinical testing must be done prior to determining the immunogenicity of engineered exosomes. Outside of health risks to patients, the safety of handling exosomes must also be considered. Fortunately, due to exosomes human origin, they should not incur any major health risks onto the researchers, physicians, and engineers that will be producing or handling the product.

6.2. Manufacturability

Because our technology is produced by human cells, the manufacturing of engineered exosomes is fairly unique. As opposed to a traditional technology, the only thing that is actually designed or engineered throughout the production of our technology is the initial DNA plasmid that directs cells how to produce our designed exosomes. So, after the DNA plasmid is uploaded into human cells, the cells will fully produce the technology. One major drawback of this is that the exact production process is not controllable. Therefore, standardizing production quality and rate is difficult and unpredictable. Additionally, human cells produce exosomes at a relatively slow rate, therefore further bioreactor technologies must be developed to allow for optimized production and isolation of engineered exosomes.

6.3. Economic

Current approaches to exosome technology limit the targeting of each engineered exosome to one specific target. With our design, disease-targeting specificity does not have to be assigned until after the exosomes are produced. Therefore, these programmable exosomes can be produced on a large scale all together instead of producing specific exosomes for very specific disease processes. Therefore, this should ease the economic burden of producing these exosomes, and hopefully, this will lower the cost of accessing this treatment option.

6.4. Sustainability

Similar to the economic implications, our suggested project should make the production of engineered exosomes easier. Therefore, there should be less waste throughout the production process. In the more traditional method, exosomes are limited in their application, therefore they may cause unnecessary waste via unused products. With our design, ideally every exosome can be used as the technology's application is broad. However, the production of exomes is a resource expensive process that produces waste. For instance, many of the materials that are used during the process are nonrecyclable, and some materials

require incineration after use due to their biohazardous properties. Furthermore, the producer cells themself produce waste and often die through the exosomes production process, and this further produces unintended waste.

6.5. Ethical

The first major ethical consideration of our technology is the access of our engineered exosomes in a therapeutic setting. Therapeutic materials are typically very expensive to access, and exosomes are no different. In fact, exosomes are especially expensive because they have to be designed and produced to treat specific disease processes. However, due to the robust application of our technology, our engineered exosomes should be cheaper and easier to access than more traditional alternatives. Additionally, the application of exosomes in a therapeutic setting requires that technology produced by one human is essentially transported into another human. Therefore, there may be similar concerns with exosomal applications as some cultures do with transplant procedures.

7. Conclusion

7.1. Summary and impact

Exosomes are a powerful, versatile nanovesicle that are naturally produced by cells and widely used for cell-to-cell communication. We have demonstrated the application of taking advantage of this naturally occurring pathway to create a novel therapeutic delivery system utilizing exosomes to bypass common biocompatibility issues associated with more widely used nanomedicine and immunotherapies. In addition, we have confirmed the feasibility of using Strep-tag to simplify the purification process for separating exosomes from other similarly sized nanoparticles found in extracellular fluid. We have yet to confirm the potential of target switching, resulting in an extremely powerful system utilizing one-design, with multiple potential disease targets.

The successful completion of this research could reveal the almost limitless potential of using this exosome technology to treat a large variety of human diseases. By targeting a known surface marker related to a disease, the engineered exosome system can directly deliver therapeutics to diseased cells, and even bypass natural barriers within the body, such as the blood-brain barrier. Additionally, the scope is not just limited to a particular subset of diseases, such as cancer, but any diseases that result in the expression of a targetable surface marker. Different types of cancers can be addressed by targeting different cancer surface markers, ranging from CD19, to PD-L1, or even Her2. At the same time, neurological diseases can be treated by targeting toll-like receptors found on neurons, or chronic inflammation, such as rheumatoid arthritis, by targeting TNFα. Lastly, the switchable interior adds an additional layer of customization, as the material encapsulated within the engineered exosome can be changed for a variety of purposes. Exosomes can deliver diagnostic material to help with imaging and surgery, drugs for therapy, or genetic material for gene editing.

7.2. Future Work

7.2.1. Confirming functionality of PNE-scFV segment

Due to the global pandemic of COVID-19, we were unable to see our project through to the completed state we had originally envisioned. The initial groundwork of this final step has already been laid, with the supplementary protein already being designed and synthesized. The first step for continuing this project should be to conduct a co-transfection in 293T cells with the exosome and PNE proteins. Both proteins were designed to be ultimately secreted from the cell, and upon successful binding, co-localization of the eGFP and rRFP signals should be visualized using confocal microscopy.

7.2.2. Creating a stable cell line

This additional step can be considered optional, as all of our exosome samples this year were harvested from transiently transfected cells. The creation of a stable cell line would allow a future group to obtain a regular supply of transfected exosomes with much less effort than having to regularly transiently transfect cells for exosome harvest. Two separate stable cell lines should be created, one with the exosome protein, and the other with the PNE protein, so that separate samples of both can be obtained.

7.2.3. Testing switching ability of PNE-scFV segment

Regardless of if a stable cell line is created or not, the next stage of the project should be to create an additional protein capable of binding with the PNE-scFV segment of the exosome protein, and determining the extent of the switching ability of the PNE-scFV itself. Additional research would heavily focus on this area, as even more traditional sCAR-T therapy encounters difficulty when switching targets.

7.2.4. Delivering therapeutics to a diseased cell

Finally, a future group can consider combining all stages of the project, and attempt to deliver therapeutics to a diseased cell using the intermediary PNE targeting system. For example, 293T cells can be transfected to express CD19 on their surface, and a new GCN4-anti-CD19 protein should be synthesized to allow the exosome system to target the CD19 expressed on the surface of the transfected 293T cells. If this targeting is successful, therapeutics can then be encapsulated within the exosome and delivered to the transfected 293T cell, and experiments should be conducted to test the efficacy of the entire system.

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