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# Engineering Nanoscale Exosomes for Lysosomal Delivery of Bioactive Enzymes

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SANTA CLARA UNIVERSITY

Department of Bioengineering

I HEREBY RECOMMEND THAT THE THESIS PREPARED  
UNDER MY SUPERVISION BY


**Mai Anh Do**

ENTITLED

**Engineering Nanoscale Exosomes for Lysosomal Delivery  
of Bioactive Enzymes**

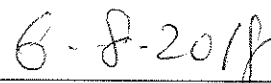
BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS  
FOR THE DEGREE OF

**Master's of Science in Bioengineering**

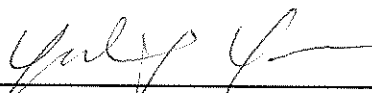


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Thesis Advisor - Biao Lu, MD/PhD



Date



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Department Chair - Yuling Yan, PhD



Date

**Engineering Nanoscale Exosomes for Lysosomal  
Delivery of Bioactive Enzymes**

By

**Mai Anh Do**

Master's Thesis

Submitted to  
the Department of Bioengineering

of

SANTA CLARA UNIVERSITY

in Partial Fulfillment of the Requirements  
for the degree of  
Master's of Science in Bioengineering

Santa Clara, California

2018

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## DISCLOSURE

This is a joint study of Mai Anh Do and Daniel Levy. Data overlap between the two submitted theses.

Figure 1 prepared by Dr. Biao Lu.

Figure 2, 4, 8-9 prepared by Mai Anh Do.

Figure 3, 5-7 prepared by Daniel Levy.

Figure captions were written on individual basis.

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# Engineering Nanoscale Exosomes for Lysosomal Delivery of Bioactive Enzymes

Mai Anh Do

Department of Bioengineering  
Santa Clara University

2018

## ABSTRACT

There are at least 50 lysosomal storage diseases that are caused by accumulation of a substrate due to a defective enzyme. Although some treatment options are available, low efficacy, high cost, and immunogenicity are main challenges of current treatments. To overcome those limitations, we use cell-derived nanoparticles to deliver biologically active cargos into lysosomes. Naturally produced exosomes can deliver proteins without triggering an immune response. Additionally, exosome have an intrinsic ability to cross blood brain barrier benefiting patients at a late disease stage that affects the brain. After fusing either *Gaussia* luciferase (gLuc) or puromycin resistant protein (puro) onto a vesicular stomatitis virus glycoprotein (VSVG) with GFP/RFP reporters, we show that exosomes can be loaded with the recombinant protein without changing basic characteristics of exosomes. Moreover, we show that the biological activity of the proteins is retained in both the producer and recipient cells at a statistically significant level. As expected, the modified exosomes co-localize with both lysosomal and endosomal compartments indicating that they still undergo an endosomal pathway after an uptake assay. This work demonstrates that we can engineer nanoscale exosomes for delivery of therapeutic enzymes into lysosomes. The engineered vesicles have a great potential of becoming a method for enzyme delivery into patients with a lysosomal storage disease.

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## INTRODUCTION

### 1.1 Background

Delivery of therapeutic proteins into cells is challenging although protein drugs have been widely used since the late 20<sup>th</sup> century.<sup>1</sup> Moreover, there are limitations to the therapeutics due to protein instability, immunogenicity, low bioavailability or specificity.<sup>1,2</sup>

There are approximately 50 different lysosomal storage diseases<sup>3,4</sup> caused by lysosomal enzymes that are either missing or expressed at a very low level. The inefficient level of enzymes leads to accumulation of substrates which can have a downstream effect on the cell pathology.<sup>4</sup> Gaucher's, Tay-Sachs, and Salla disease are examples of lysosomal storage diseases that exhibit different debilitating forms in infants, juveniles and adults.<sup>5</sup> There are many approaches to treat lysosomal storage diseases such as enzyme replacement therapy or substrate reduction therapy<sup>6</sup>. In this work, we will focus on a new approach that takes advantage of cell-derived nanoparticles called exosomes to deliver therapeutic proteins into cells.

Exosomes are nanovesicles ranging between 30-150 nm in diameter. They are composed of a phospholipid bilayer membrane similar to a plasma membrane. Exosomes are naturally produced, loaded, and secreted by all human cells.<sup>7,8</sup> Exosome production starts with invagination of plasma membrane to form an intracellular compartment called endosome.<sup>9</sup> When endosomal membrane undergoes another round of invagination, smaller intraluminal vesicles are formed (Fig. 1C). An endosome containing multiple intraluminal vesicles is called a multivesicular body (MVB) as seen in Figure 1C. Usually, the MVB then fuses back with the plasma membrane releasing the content into the extracellular space. Once released, the intraluminal vesicles are called exosomes.<sup>9</sup> Another pathway for MVB is through fusion with lysosome where the released content is either destroyed<sup>10</sup> or used as a potential enzyme replacement. The primary purpose of exosomes is cell communication since the exosomal lumen is loaded with cytoplasmic content of the producer cell during formation.<sup>9,10,11</sup> Different RNA and protein cargos are delivered after exosomes are endocytosed into a recipient cell as shown in Figure 1D. During this process, exosomes end up back in the endosomal compartment where they either fuse back and release the content to the cytoplasm, or are delivered into lysosomes.<sup>9</sup> Since exosomes are produced naturally by our cells, they have a greater potential of delivering drugs without triggering immune response compared to synthetic nanoparticles. Many attempts of using exosomal pathway for drug delivery have been made. Exosomes have been shown to successfully deliver siRNAs both in vitro and in vivo<sup>12,13</sup>. They can also be loaded with proteins, lipids, and DNA<sup>14,15</sup> which demonstrates their immense potential.

### 1.2 Current Technologies and Motivation

Currently, there are several available treatments for lysosomal storage diseases, however, the technologies are very limited due to low efficacy, high cost, or impracticability. One possible strategy is a bone marrow transplantation<sup>16</sup> that uses hematopoietic progenitor cells to produce the missing enzyme. This treatment is, however, only possible for young children under three years old with an available donor,<sup>6,17</sup> which makes most of the lysosomal storage disease patients ineligible. Another strategy is an enzyme replacement therapy<sup>18,19</sup> where patients are given an infusion with functional enzyme, however, the bioavailability of most enzymes is very low which



leads to repeated infusions. Moreover, naked enzymes do not have the ability to cross a blood brain barrier, and thus, the therapy is limited to patients without mental retardation. In substrate reduction therapy, it is required that the patient has residual enzyme activity, which makes the treatment unsuitable for patients with zero active enzyme level.<sup>20,21,22</sup> If a patient produces the required enzyme in a misfolded form, chemical chaperone therapy<sup>23,24,25</sup> can be used to stabilize the enzyme. Again, this therapy is limited because only patients with minimal enzyme expression can undergo the treatment. In the recent years, gene therapy<sup>18,26</sup> for lysosomal storage diseases started to be explored. Nonetheless, the treatment is expensive and not effective in all patients. Gene therapy for a lysosomal storage disease of the central nervous system is also not well understood.<sup>6</sup>

Low bioavailability, impracticality, and inability to surpass blood brain barrier are the main limitations of current treatments. Recent studies have yielded new strategies for overcoming the current drawbacks by using nanoparticles to deliver missing proteins into lysosome.<sup>27</sup> Nanoparticles can provide targeted drug delivery, improve drug stability, and reduce side effect.<sup>28</sup> However, most nanoparticles are synthetic, therefore, there is a great chance of triggering patient's immune system even with immunosuppression treatment.<sup>28</sup>

In this study, we use cell-derived nanoparticles to deliver missing bioactive enzymes into lysosomes. Exosomes have the ability to be loaded with any protein, shuttle it from a producer to recipient cell, and eventually unload the cargo into lysosome. Since exosomes are naturally produced by human cells, there is very low probability of immune response. Moreover, exosomes have an innate ability to cross a blood brain barrier, therefore, making it possible to treat all stages of lysosomal storage diseases. Exosomes could also be engineered to deliver drugs into targeted tissue by changing the outer membrane profile.

To load our therapeutic protein into exosomes, we use an anchoring transmembrane protein. The most common membrane proteins for extracellular vesicles including exosomes are CD63, CD81, and CD9.<sup>29</sup> These tetraspanins have cytoplasmic, transmembrane, as well as extracellular domains, making them potentially good anchors for the cargo. However, loading onto these proteins can be quite challenging because of the size and structure of the proteins. Here, we use vesicular stomatitis virus glycoprotein (VSVG) that contains the same domains as the CD proteins but VSVG penetrates the membrane only once. Additionally, it has been shown that this viral envelope protein increases the loading ability of the cargo during transfection<sup>30,31</sup> with exception to some immune cells.<sup>32</sup> VSVG has been approved for various clinical trials due to its high efficacy.<sup>30</sup> Here, we fuse VSVG with *Gaussia* luciferase (gLuc) enzyme due to its simple and robust activity assay.<sup>33,34</sup> In addition to the therapeutic protein, we also attach GFP or RFP as visual reporters for tracking and analysis.

### 1.3 Project Goals

To deliver biologically active proteins into lysosome using exosomes, we have a four-fold objective. First, we have to design and engineer the DNA vector containing a fusion of VSVG, protein of interest (gLuc or puro), and visual reporters (GFP or RFP). Next, we transfect the constructs in the cells to track the expression and production of the fusion proteins in the intraluminal vesicles. Third, we characterize exosomes outside the cell and show biological activity. Lastly, we deliver the cargo-containing exosomes into recipient cells and show co-localization with lysosome, as well as retained biological activity.

## MATERIALS AND METHODS

### 2.1 Design and Construction of VSVG Fusion Proteins

The expression vector for the VSVG fusion protein contains a CMV promoter, VSVG coding sequence, GFP or RFP coding sequence, *Gaussia* Luciferase (gLuc) or Puromycin resistance gene sequence (puro), and a polyA signal as shown in Figure 1A. All sequences were attached on the C-terminus of VSVG which allowed for loading the cargo proteins into the exosomes as shown in Figure 1B. The construction and DNA sequencing of the vector was carried out at GenScript, CA, USA. In this paper, GFP/RFP will be referred as reporter, while puro/gLuc as cargo.

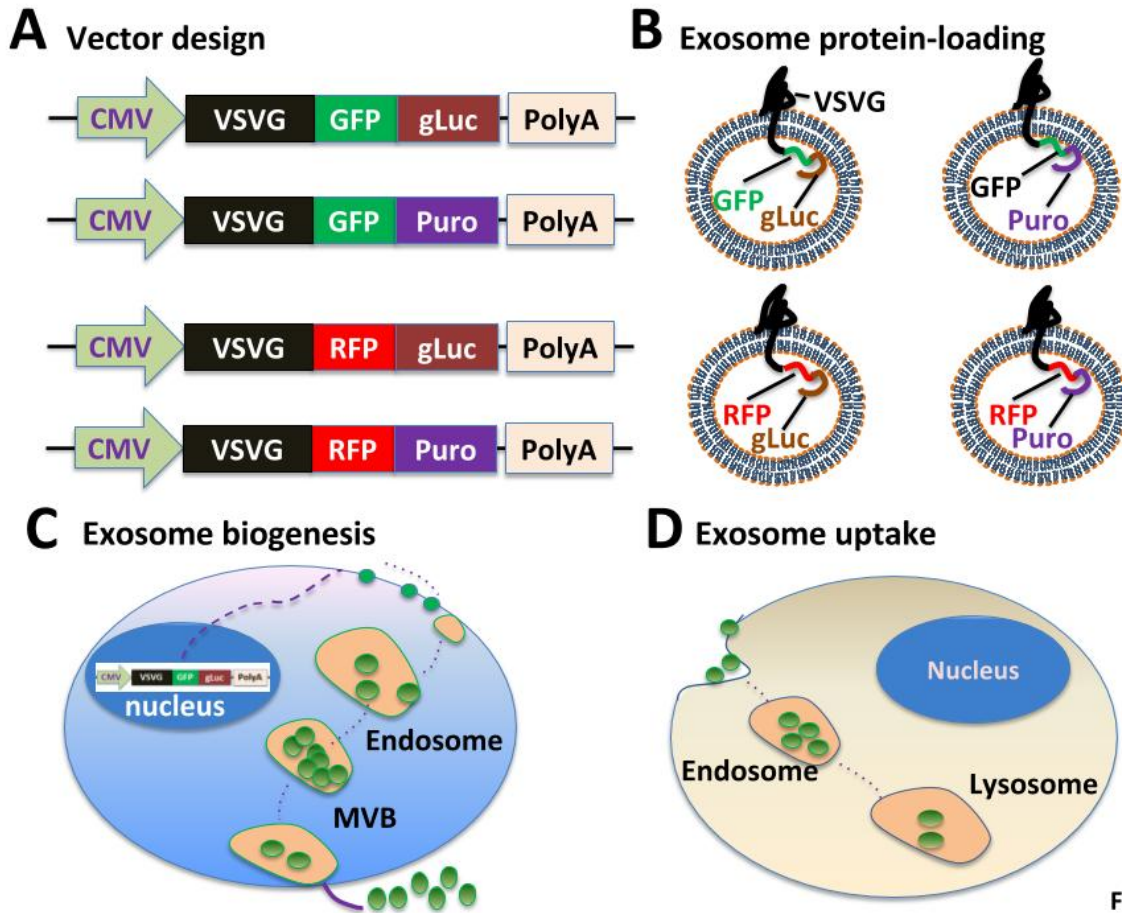


Figure 1

**Figure 1. Design and construction of dual tagged reporter protein for exosome tracking.**

(A) Vector design for VSVG fusion proteins. Fluorescent (GFP, RFP) and activity (gLuc, puro) reporter genes were respectively attached at the C-terminus. (B) The schematic of dual loaded exosomes showing VSVG (black) transmembrane protein, GFP (green) or RFP (red) reporters, and gLuc (brown) or Puro (purple) protein cargos. (C) Illustration of VSVG-exosome biogenesis. Expressed VSVG-reporter-cargo construct is transferred to plasma membrane. Inward budding of the VSVG-reporter-cargo enriched plasma membrane creates endosomes. Once endosomes are formed, exosomes are generated by inward budding of the endosomal membrane. At the late-endosome stage, multivesicular body (MVB) fuses with plasma membrane resulting in exosome release into extracellular space. (D) Proposed exosome uptake model. Cells incorporate extracellular exosomes into the endosomes through endocytosis. Then, the endosomes unload the cargo at a final destination (lysosome).

## **2.2 Cell Culture and Transfection**

HEK293 cells (Alstem, CA, USA) were cultured in Dulbecco's Minimal Essential Medium (DMEM) (Gibco, MA, USA) supplemented with 10% fetal bovine serum (FBS) (GE Healthcare Life Sciences, PA, USA) and 1% Penicillin Streptomycin (Pen Strep) (Gibco, MA, USA). Human hepatocellular carcinoma cells (HEPG2), human glioblastoma cells (U87), and mouse adipose tissue fibroblast cells (L929) were purchased from American Type Culture Collection (VA, USA) and cultured under the same condition. All cells were maintained at 37°C with 5% CO<sub>2</sub> in humidified air. At ~80 % confluence, cells were passaged using 0.25% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) (Gibco, MA, USA) for cell dissociation. At 40, 50, or 60% confluence, cells were transfected in a 6-well plate using plasmid DNA (2 µg/well) and 10 µl polyethylenimine (PEI) transfection reagent. DNA and PEI were each diluted in 100 µl of reduced serum media Opti-Mem (Gibco, MA, USA) and mixed. Transfection complex was incubated for 20 min then added into cell culture. For co-transfection, 1 µg of each DNA was used per well. For exosome preparation, 20 µg of DNA, 100 µL of PEI, and 2 ml of Opti-Mem was used per 145mm culture dish.

## **2.3 Live Cell and Exosome Microscopy**

Live cell or exosome images were taken using a Lumen Dynamics x-cite 120LED Olympus CKX53 fluorescent microscope and processed through Olympus cellSens Standard software. Leica TCS SP8 confocal laser scanning microscope and Las X software were used to capture and process confocal images. Images of the same field were taken under phase contrast, and GFP and/or RFP settings at 20x or 40x magnification. Pictures were overlaid using GIMP 2.8.22 software to show GFP and/or RFP location.

## **2.4 Exosome Preparation and Isolation**

DMEM (+10% FBS and 1% PenStrep) media change to serum free UltraCulture (Lonza, Switzerland) was done on HEK293 day one post transfection or two days post stable cell line passage. After 48 hours, conditioned culture media was collected from 145 mm culture dishes and centrifuged for 10 min at 1500g. The supernatant was then filtered through a 0.2 µm polyethersulfone membrane filter (VWR, PA, USA). Exosomes were precipitated from the supernatant by adding ExoQuick-TC (System Biosciences, Inc., CA, USA) in 1:4 ratio, and incubated overnight at 4°C. Exosomes were collected by centrifuging at 3000g for 1.5 h. The pellet was resuspended in 150-300 µL PBS and final concentration was determined using NanoDrop Lite (Thermo Fisher Scientific, CA, USA). Purified exosomes were kept in 4°C for immediate use or stored at -20°C for future use.

## **2.5 Drug Selection and Stable Cell Line Establishment**

24 h post transfection, HEK293 cells were treated with 5 µg/ml puromycin dihydrochloride (Thermo Fisher Scientific, CA, USA) in DMEM (+10% FBS and 1% PenStrep). Puromycin media was regularly changed until cells became stable. Stable cell line was established when cells appeared healthy, attached, and GFP/RFP-positive 65 days post puromycin treatment. Cells were

kept without puromycin pressure for at least one week before conducting further experiments.

## 2.6 Luciferase Activity Assay

500  $\mu$ L (30  $\mu$ L) of 1x Reporter Lysis Buffer (Promega, WI, USA) per well was used to lyse cells in a 6-well (96-well) plate 24 and/or 48 h post transfection, or 3, 8, 24, 48, 72 h post exosome uptake. 20  $\mu$ L of lysed cells or purified exosomes was used per 100  $\mu$ L of coelenterazine *Gaussia* luciferase substrate. The assay was performed in a white flat bottom 96-well plate without cover. Luminescence was measured on a TECAN infinite M200PRO plate reader using TECAN i-control 1.8 software with 10 s exposure time.

## 2.7 Exosome Uptake Assay

HEK293, U87, L929 cells were cultured in a 96-well plate or a 4-chamber glass bottom plate until they reached 70 % confluence. Exosome dilution of 0.3  $\mu$ g/ $\mu$ l in serum free UltraCulture (Lonza, Switzerland) was prepared and media change was performed on the day of uptake assay. For VSVG-GFP-gLuc or VSVG-RFP-gLuc exosomes, cells were washed 3x with pre-warmed PBS before lysing with 1x Reporter Lysis Buffer (Promega, WI, USA). Imaging or luciferase assay was followed at corresponding time points.

## 2.8 HOECHST Staining

1 mg/mL of Hoechst 33342 Stain (Thermo Fisher Scientific, IL, USA) solution was prepared in PBS. Media change was performed using the stain solution diluted 1:1000 in PBS. Plates were covered in aluminum foil and incubated for 10 min at 37°C with 5% CO<sub>2</sub> in humidified air. Cells were washed 1x using pre-warmed PBS. Cells were kept in PBS for imaging using confocal microscopy.

## 2.9 Western Blot

15 or 60  $\mu$ g of each exosome construct was reduced with 3x SDS sample buffer (New England BioLabs, MA, USA) containing of 0.1 M DTT reducing reagent in 2:1 ratio and then incubated for 5 min at 90 °C water bath. All samples and 7  $\mu$ L of Prestained Protein MW Marker #26612 (Thermo Fisher Scientific, CA, USA) were then loaded into each well of a 4-12% ExpressPlus PAGE gel (GenScript USA Inc, NJ, USA). Electrophoresis was run for 1 h at 100 V. After electrophoresis, the gel and two sheets of filter blotting paper (ThermoFisher Scientific, CA, USA) were incubated at RT in diH<sub>2</sub>O for 10 min, followed by incubation at RT in 25mM tris/192mM glycine transfer buffer (BioRad, CA, USA) containing 20 % lab grade methanol (Fisher Science Education, PA, USA). Amersham Hybond P 0.2  $\mu$ m PVDF membrane (GE Healthcare Life Sciences, CA, USA) was activated by incubation in methanol for 20 s, diH<sub>2</sub>O for 20 s, and then transfer buffer for 10 min. Proteins were transferred onto the membrane using a semi-dry transfer cell (Bio-Rad, CA, USA). The transfer was run at 12 V for 12 min. The membrane was then incubated in 5 % non-fat dry milk (LabScientific, NJ, USA) 1x TBST pH 7.6 (TEKNOVA, CA, USA) blocking buffer for 1 h at RT rocking. The membrane was then incubated in CD81 (Santa Cruz Biotechnology, CA, USA) or CD9 (abcam, Cambridge, UK) monoclonal mouse primary antibodies in a 1:500 ratio in blocking buffer. CD63 and HSP70 monoclonal rabbit antibodies (System Biosciences, CA, USA) were used in 1:1000 ratio. The overnight incubation

was carried at 5°C rocking. Following the primary incubation, the membrane was washed 3 x with 1X TBST for 5 min. The goat anti-mouse (Thermo Fisher Scientific, IL, USA) or anti-rabbit (System Biosciences, CA, USA) HRP-conjugate secondary antibody was diluted 1: 2,500 and 1: 10,000 in blocking buffer and membrane was incubated for 1 h at RT rocking. The membrane was washed 3 x with 1X TBST for 5 min before visualization. Part I and II of Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, CA, USA) were mixed in 1:1 ratio and the membrane was incubated in the substrate solution for 1 min at RT rocking. The membrane was transferred into a plastic wrap and visualized using the ImageQuant LAS 500 (GE Healthcare Life Sciences, CA, USA) under chemiluminescence setting and 1 h exposure.

## **2.10 Dot Blot**

250 µg of harvested VSVG-RFP-gLuc and VSVG-RFP-puro exosomes were sent to System BioSciences, CA, USA for Dot Blot Analysis.

## **2.11 ELISA**

ExoELISA-ULTRA protein standard (System Biosciences, CA, USA) was used to establish a protein standard curve. Exosome samples were diluted to 120 µL with Coating Buffer (System Biosciences, CA, USA). 50 µg of exosomes was used per each ExoELISA micro-titer plate (System Biosciences, CA, USA) well. The plate was sealed and incubated at 37 °C for 1h. The plate was washed 3x for 5 min each with 100 µL of Wash Buffer (System Biosciences, CA, USA). 50 µL of CD63 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 in Blocking Buffer (System Biosciences, CA, USA) was added to each well. The plate was incubated at RT for 1 h shaking followed by 3x wash for 5 min each with 100 µL of Wash Buffer. 50 µL of secondary antibody diluted 1:5,000 in Blocking Buffer (System Biosciences, CA, USA) was added to each well. The plate was incubated at RT for 1 h shaking followed by 3x wash for 5 min each with 100 µL of Wash Buffer. Next, 50 µL of Super-sensitive TMB ELISA substrate (System Biosciences, CA, USA) was added to each well and the plate was incubated at RT for 15 min shaking. Finally, 50 µL of Stop buffer (System Biosciences, Palo Alto, CA, USA) was added followed by immediate quantitation using spectrophotometric plate reader at 450 nm.

## **2.12 Nanoparticle Tracking Analysis**

Approximately 150 µL of 3-4 µg/µL harvested exosomes from all constructs and plain HEK293 cells were sent to Particle Characterization Laboratories, Inc. Novato, USA for NTA analysis.

## **2.13 Lysosomal Staining**

LysoTracker Red DND-99 (Invitrogen, Oregon, USA) stain was diluted in DMEM media to the final concentration of 75 nM. Media was changed to the pre-warmed staining solution and cells were incubated for 30 min at 37°C with 5% CO<sub>2</sub> in humidified air. Media change to regular DMEM was performed before imaging cells using confocal microscopy.

## **2.14 Endosomal Staining**

10 µL of either CellLight Early Endosomes-RFP (Invitrogen, Oregon, USA) or CellLight Late Endosome-RFP (Invitrogen, Oregon, USA) stain was added into HEK293 cells 48 hours after

VSVG-GFP-puro exosome uptake. The cells were incubated in the staining solution for 16 hours at 37°C with 5% CO<sub>2</sub> in humidified air, and visualized using confocal microscope. Staining was performed in a 96-well plate.

### **2.15 Cell Counting**

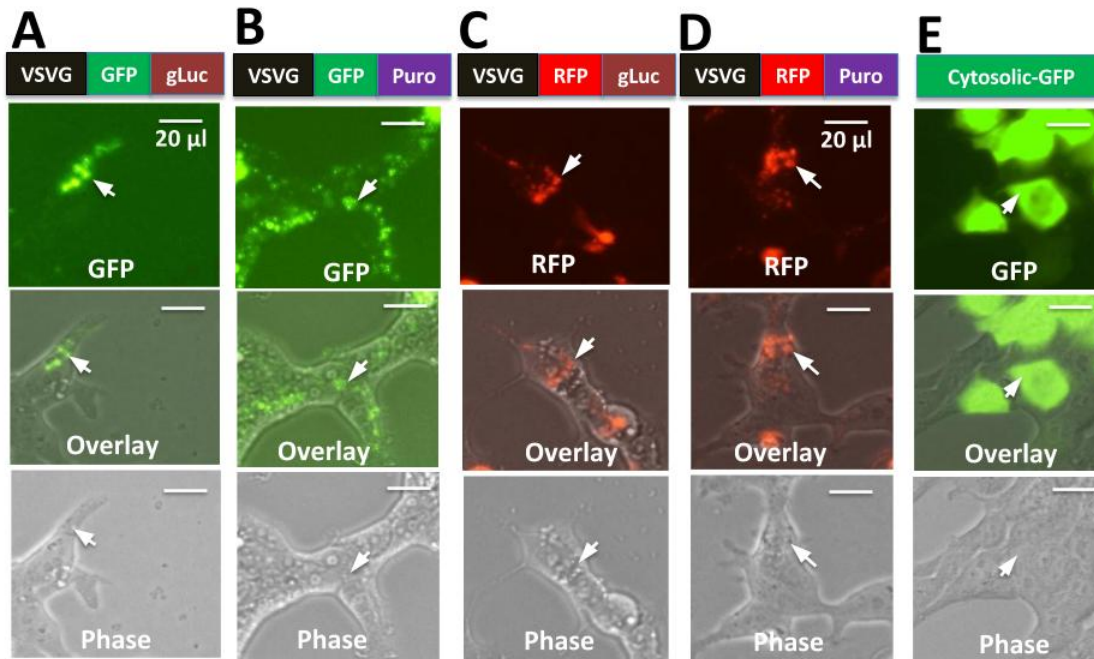
Cell counting was performed on cells treated with modified exosomes for 48h and puromycin for additional 48h. Cell media from each well of a 96-well plate was collected into separate 1.5ml microcentrifuge tubes. Cells were detached using 25 µL of 0.25% trypsin-EDTA (Gibco, MA, USA) and collected in the same microcentrifuge tubes. Cells were centrifuged at 1500x g for 5 min and re-suspended in 100 µL of DMEM (+10% FBS and 1% PenStrep). 1:1 ratio of cell suspension to Trypan Blue 0.4% Solution (MP Biomedicals, LLC, Ohio, USA) was mixed. Cells were then counted on a C-Chip Hemocytometer (INCYTO Co., Ltd., Convington, GA) under a Vista Vision light microscope (VWR, Brisbane, CA).

## RESULTS

### 3.1 Results

#### *Intracellular Tracking of VSVG Constructs in HEK293 Cells*

After HEK293 cell transfection with VSVG constructs, cells were tracked at three time points. Starting 24 hours post transfection, formed intraluminal vesicles (exosomes) contained the engineered VSVG-reporter-cargo constructs as indicated by the punctuated morphology. The modified-exosome containing cells were tracked again 48 and 72 hours (Fig. 2) post transfection. As predicted, the morphology of the intraluminal vesicles from all constructs did not change with time, which was compared to the morphology of cytosolic GFP that was evenly distributed throughout the cell.

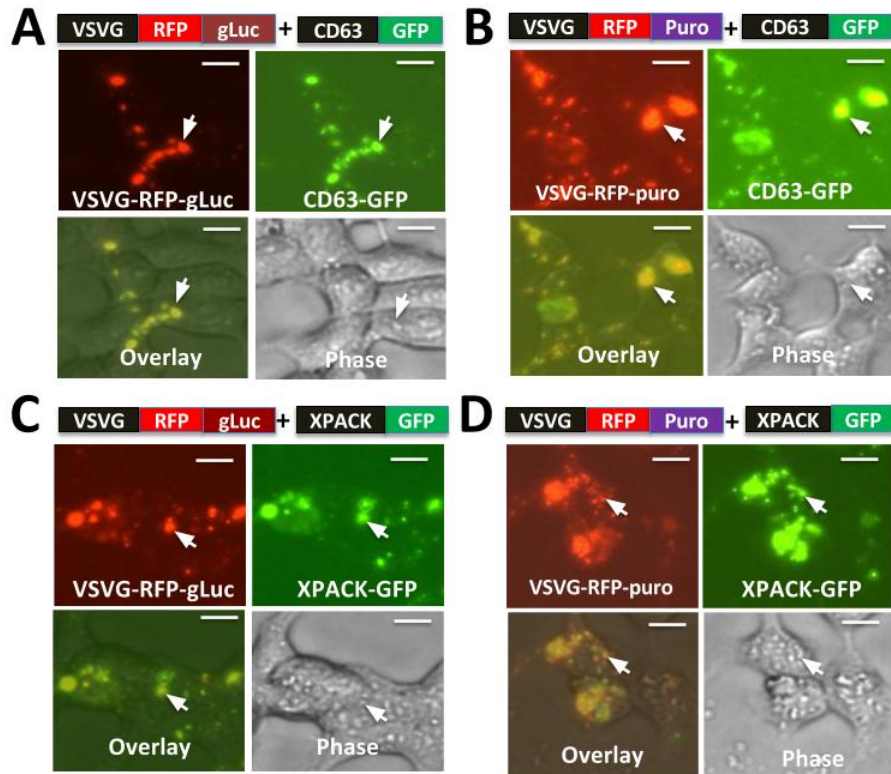


#### **Figure 2. Intracellular tracking of exosome biogenesis.**

Formation of modified exosomes in HEK293 cells was tracked at 24 (not shown), 48 (not shown), and 72 hours (A-D) post PEI transfection. All 4 VSVG-reporter-cargo constructs showed punctuated formation of exosomes (A-D) compared to the disperse cytosolic GFP control (E). Negative control (not shown) had no color expression detected. Images were taken at 20x magnification using fluorescent microscope, then overlaid in GIMP software.

Following transfection, a co-transfection experiment with exosomal markers CD63 and XPACK further showed that the engineered constructs go to exosomes. As Figure 3 demonstrates, the VSVG-RFP-gLuc construct co-localized with both CD63-GFP and XPACK-GFP which was clearly demonstrated by the yellow color in the overlaid pictures (arrows in Fig. 3A, 3C). The same was achieved for the VSVG-RFP-puro construct (Fig. 3B, 3D).





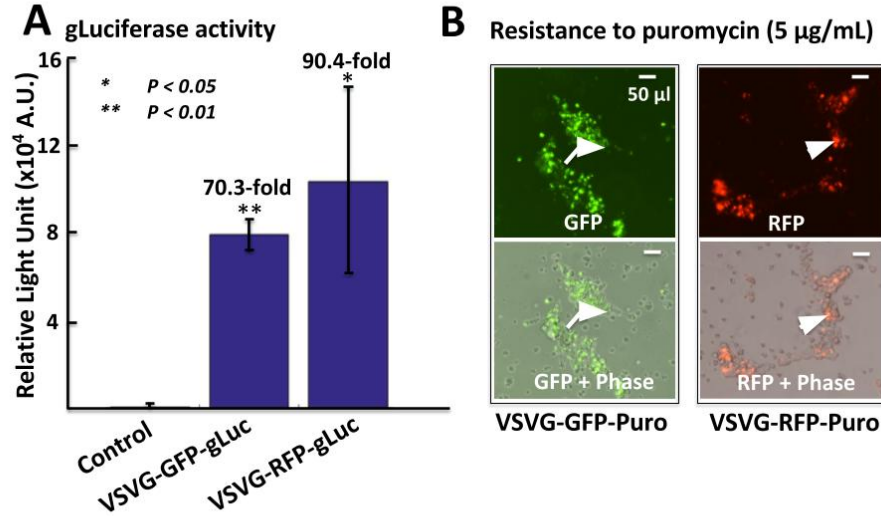
**Figure 3. Colocalization of tracking molecules with exosome markers.**

PEI co-transfection of VSVG-RFP-cargo and CD63-GFP exosome marker (A-B) in HEK293 cells showed colocalization of the constructs (yellow). The same trend was observed when the experiment was repeated with XPACK-GFP exosome marker (C-D). All plasmids were co-transfected in 1:1 ratio. Images were taken at 20x magnification using fluorescent microscope at 48 (not shown) and 72 hours (A-D) post co-transfection.

### **Biological Activity of the Cargo in the Producer Cells**

For both VSVG-GFP-gLuc and VSVG-RFP-gLuc transfected HEK293 cells, a *Gaussia* luciferase assay was performed on a cell lysate to show biological activity of the gLuc enzyme in the intracellular exosomes. 72 hours post transfection, cells containing VSVG-GFP-gLuc modified exosomes had a statistically significant 70.3-fold increase in enzymatic activity (Fig. 4A, second bar) compared to unmodified HEK293 cell lysate (Fig. 4A, first bar). The VSVG-RFP-gLuc intracellular exosomes produced a statistically significant 90.4-fold increase in the same enzymatic assay (Fig. 4A, third bar), which demonstrated the retained activity of the gLuc cargo. To test the cargo activity in the VSVG-GFP-puro and VSVG-RFP-puro modified exosomes, the transfected HEK293 cells underwent a 5 µg/ml puromycin treatment. Images taken 24 hours post treatment show that most cells were still attached and resistant to puromycin, indicating the puromycin resistant gene was still biologically active for both VSVG-GFP-puro and VSVG-RFP-puro constructs (Fig. 4B) after being loaded into exosomes.



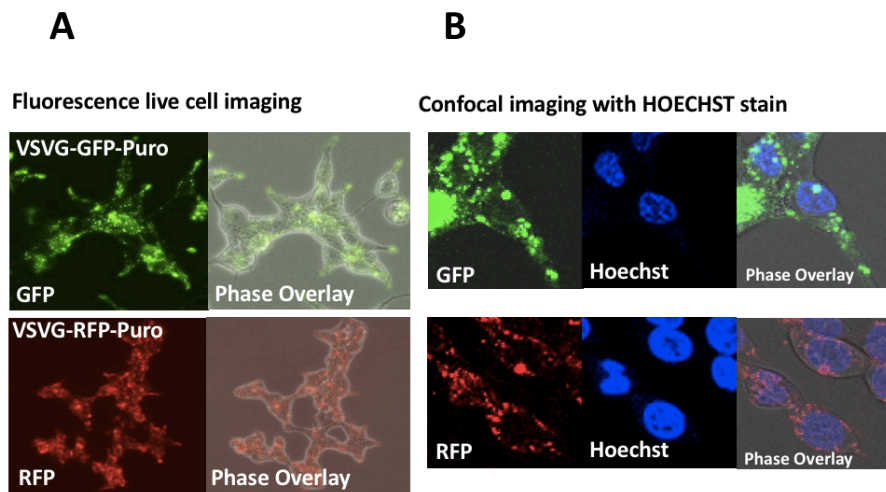


**Figure 4. Engineered tracking markers are biologically active.**

72 hours post transfection, *Gaussia* Luciferase Assay on HEK293 cell lysate showed a 70.3-fold (VSVG-GFP-gLuc) and 90.4-fold (VSVG-RFP-gLuc) increase in gLuc activity compared to the negative control (A). The assay was run in triplicate with corresponding statistical significance (\*) indicated (A). Images of HEK293 cells containing VSVG-reporter-puro construct were taken 24 hours post 5  $\mu$ g/ml puromycin treatment (B). Most cells were attached indicating biologically active protein cargo (B). Puromycin treatment of untransfected HEK293 cells (not shown) resulted in cell death within 24 hours. Images were taken at 10x magnification using fluorescent microscopy.

### Harvest and Characterization of Modified Exosomes

Modified exosomes from producer HEK293 cells were harvested from cell media for further characterization. To reduce the necessity for transient transfection of VSVG-reporter-puro constructs into producer cells, stable cell lines were established as shown in Figure 5. The characteristic punctuated morphology of exosomes was seen in both VSVG-GFP-puro and VSVG-RFP-puro modified cells (Fig. 5A) under the fluorescent microscope 40 days (GFP) or 20 days (RFP) post 5  $\mu$ g/mL puromycin treatment. Images of Hoechst stained stable cell lines were taken on a confocal microscope 72 days (GFP) or 56 days (RFP) after treatment, respectively. As seen in Figure 5B the modified exosomes were still expressed.

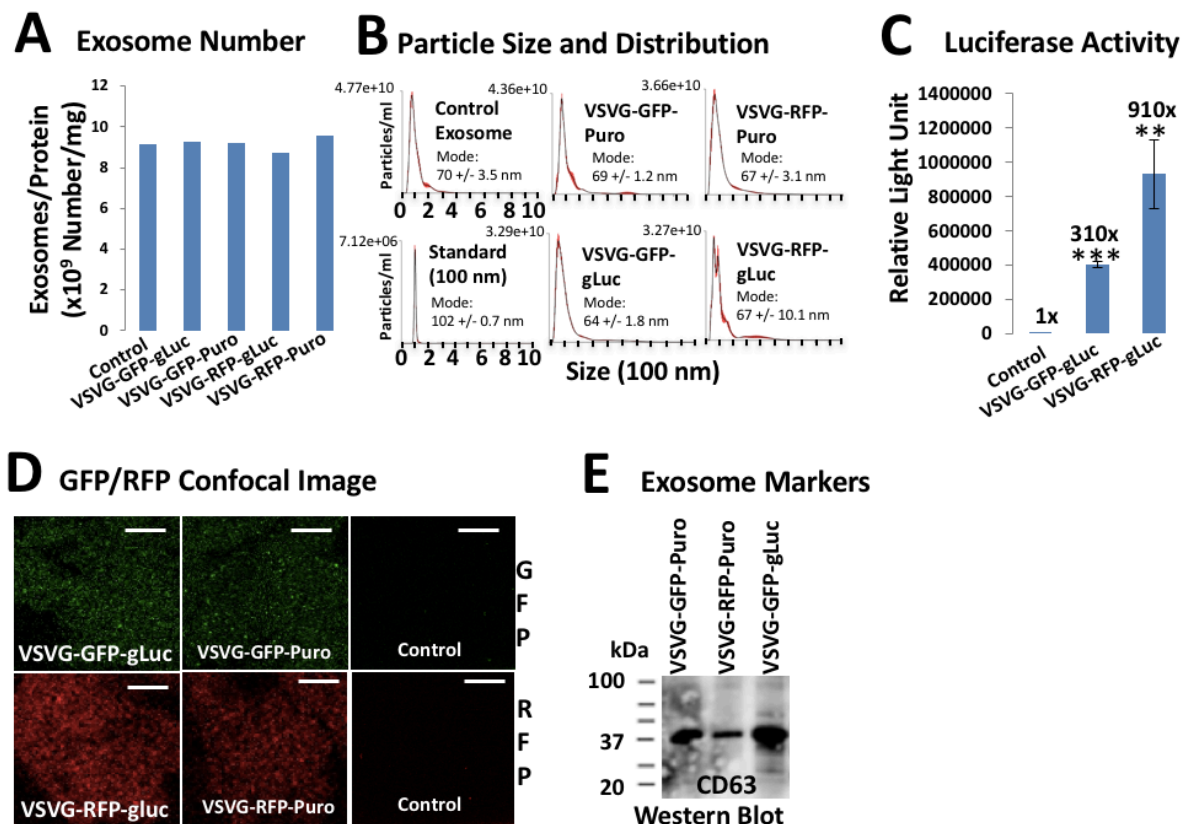


**Figure 5. Intracellular localization of modified exosomes in stable cell line.**

Puromycin treatment of 5  $\mu$ g/mL was utilized to establish a stable cell line. (A) Images of VSVG-GFP-puro (Day 40)

and VSVG-RFP-puro (Day 20) stable cell lines were taken at 20x magnification on a fluorescent microscope. **(B)** Stable cell lines were HOECHST stained. Images were taken on Day 56 (RFP) and Day 72 (GFP). Confocal microscope at 40x magnification was used.

ELISA against CD63 exosome marker was performed to quantify the harvested exosomes. All four harvests yielded on average  $9 \times 10^9$  exosomes/mg, which was in the same range as the number unmodified control exosomes (Fig 6A). Next, nanoparticle tracking analysis (NTA) was performed to characterize size distribution of the harvested exosome. All constructs had a mode between 64 – 69 ( $\pm 1.2 - 10.1$ ) nm which was comparable to the size of control exosomes  $70 \pm 3.5$  nm (Fig 6B). The results indicated that the modification of exosomes did not disturb the size distribution of the exosomes. All modified and control exosomes were also compared to the standard nanoparticle size of 100 nm (Fig. 6B). To check the cargo activity in harvested exosomes, *Gaussia* luciferase assay was performed. It was confirmed that luciferase enzyme within the exosomes was still biologically active shown by an activity increase in both VSVG-GFP-gLuc and VSVG-RFP-gLuc constructs compared to unmodified control exosomes (Fig. 6C). There was a statistically significant increase of 310 and 910 fold, respectively. The presence of GFP and RFP reporters was confirmed using confocal microscopy. As shown in Figure 6D, all four types of modified exosomes were GFP or RFP positive compared to unmodified control exosomes. Finally, a Western Blot was performed to detect exosomal marker CD63 within the modified exosomes. The results indicated the presence of glycosylated CD63 at 40 kDa in all 3 tested constructs (Fig. 6E).



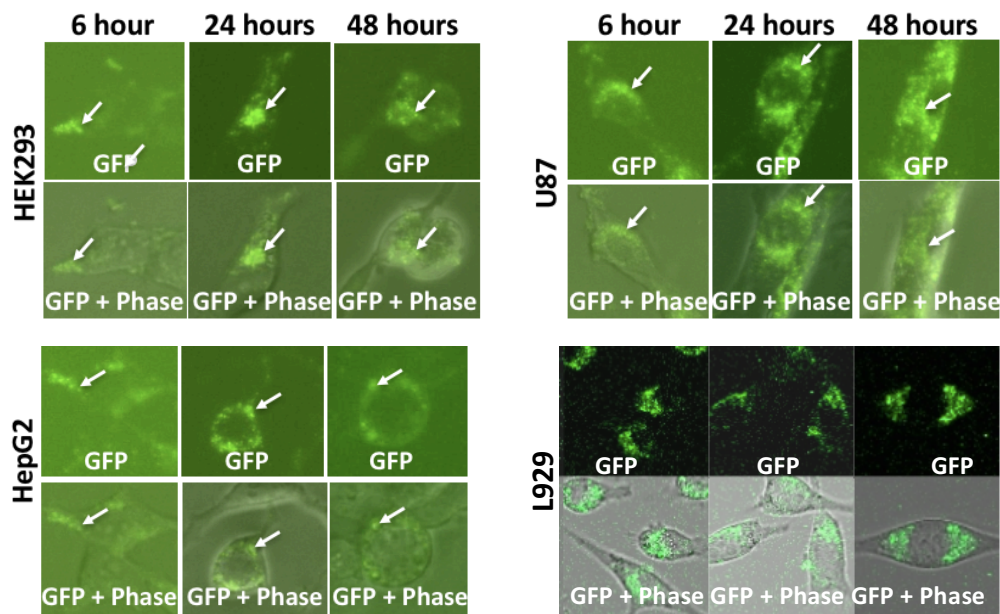
**Figure 6. Characteristics of engineered exosomes released from the producing cells.**

Engineered exosomes were purified from cell serum-free Ultra-Culture media (Gibco). Exosomes were then precipitated from the solution using ExoQuick-TC (SBI). **(A)** The amount of exosomes per milligram of protein was quantified using ELISA and antibody against CD63 exosome marker. All VSVG constructs had on average  $9 \times 10^9$  exosomes/mg which is

comparable to unmodified exosome number (control). **(B)** Exosome size and distribution was analyzed using NTA (Particle Characterization Laboratories, Inc. Novato) and compared to unlabeled (standard) and CD63 labelled (control) exosomes. All VSVG modified exosomes showed a size distribution range of 45-193 nm with corresponding modes shown. **(C)** Biological activity of loaded cargo was tested with *Gaussia* luciferase assay on purified exosomes. VSVG-GFP-gluc exosomes showed 310 x increase and VSVG-RFP-gLuc showed 910 x increase in enzymatic activity compared to unmodified exosomes (control). **(D)** Purified exosomes were observed under confocal microscope at 40 x magnification (white bar corresponds to 10  $\mu$ m) **(E)** Western Blot on lysed exosomes was performed to detect exosomal markers CD63 (and CD9 if we perform it). In all 3 tested constructs, chemiluminescence showed a band at approximately 40 kDa, which corresponds to glycosylated CD63 protein.

### ***Exosomes Uptake by Host Cells***

An uptake assay was performed to demonstrate that the harvested modified exosomes could be endocytosed by different mammalian cells. The time course tracking of exosomes showed their uptake at the edges of the cell at a 6h time point (Fig. 7) Then, the exosomes moved to paranucleus where they stayed until the final 48h time point (Fig. 7). The uptake was tested in 3 human cell lines (HEK293, Hep-G2, U87) and a mouse cell line (L929) (Fig. 7), all of which showed a successful uptake and similar distribution mechanism. The results indicate that the modification on exosomes does not hinder their natural ability to be used as a delivery vesicle.

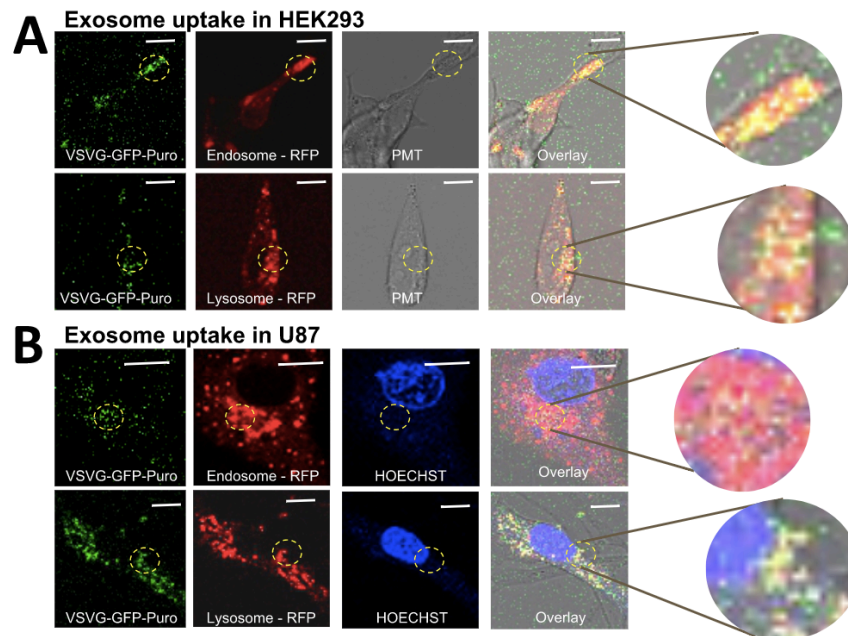


***Figure 7. Protein-loaded exosomes can be effectively taken up by mammalian cells.***

An uptake assay in four different cell lines (HEK293, U87, HepG2, L929) was performed using modified exosomes in serum-free Ultra-Culture media (Gibco). 6 hours post assay, VSVG-GFP-puro loaded exosomes were taken up as seen in the punctuated morphology on the edges of the cells (white arrows). 24 and 48 hours post assay, exosomes moved to the paranuclear space and remained at the location. Images were taken on a fluorescent microscope at 20x magnification (HEK293, U87, HepG2) or on a confocal microscope at 40x magnification (L929).

## ***Lysosomal and Endosomal Colocalization of Modified Exosomes***

After confirming that the modified exosomes could be uptaken by different cell lines, we tested if the exosomes co-localize with lysosomal and endosomal compartments. Endosomal and lysosomal staining was done on recipient cells after an exosome uptake. As Figure 8 shows, after overlaying the image of VSVG-GFP-puro construct with either endosomal or lysosomal RFP stain, there were many overlapping areas (yellow) that indicated colocalization of the modified exosomes with endosomes and lysosomes in HEK293 (Fig. 8A) and U87 (Fig. 8B) cells. The insets show enlarged overlay areas to further imply the location of the constructs in the compartments. Hoechst stain on U87 cells was performed to emphasize the nucleus (Fig. 8B).



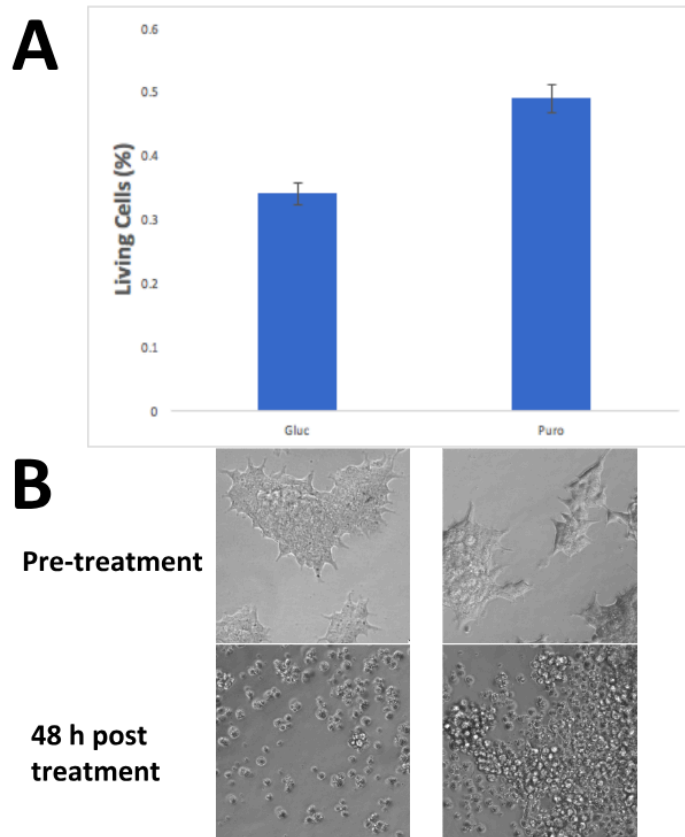
### ***Figure 8. Lysosomal and endosomal staining of exosome host cells.***

48 h after VSVG-GFP-puro exosome uptake, either HEK293 (A) or U87 (B) cells were stained using LysoTracker Red DND-99 (Invitrogen) lysosomal stain or CellLight Early Endosome-RFP (Invitrogen) endosomal stain as indicated. Cells stained for lysosome were incubated at 37°C with 5% CO<sub>2</sub> for 30 min whereas cell stained for endosomes were incubated for 16 hours under the same conditions. Overlaid images and insets show yellow colocalized areas where VSVG-GFP-puro is implied to be in the compartments. Confocal images were taken at 40 x magnification. White bars correspond to 10 μm.

## ***Biological Activity of the Cargo in the Host Cells***

Finally, we tested biological activity of the cargo after cell uptake. For VSVG-reporter-puro construct, a cell counting assay was done 48 hours after puromycin treatment to determine the number of live cells. As data show, there were more live cells in the well with puro modified exosomes (50 %) compared to the gLuc (35 %) modified control well (Fig. 9A). Both wells were then compared to a control well that did not undergo puromycin treatment. The data gained from the counting assay was supported by the images taken before treatment and 48 hours after treatment (Fig. 9B). For VSVG-reporter-gLuc construct, the enzymatic activity increased with time in cells that underwent uptake treatment with VSVG-reporter-gLuc modified exosomes (data not shown). After 48 hours, this activity decreased.





**Figure 9. Biological activity of puromycin resistant protein after exosome uptake.**

VSVG-GFP-cargo modified exosomes were added into HEK293 cells. After 48 hours, cell counting was done on the gLuc and puro groups. The percentages of live cells in the gLuc group was 35% and puro group 50% both compared to a control that was not treated with puromycin (A). Images were taken prior as well as 24 h (data not shown) and 48 h (B) after 3 $\mu$ g/ml puromycin treatment. Images show that most cells in the gLuc group were dead whereas puro group still had some live cells attached. Images were taken on a florescent microscope at phase setting.

### 3.2 Discussion

Although there are many approaches for delivery of therapeutic proteins into lysosomes, all of them have limitations including immunogenicity, low bioavailability, or inability to cross blood brain barrier.<sup>1,2</sup>

To overcome the limitations, we have combined nanoparticle technology to deliver proteins by designing four vectors for a VSVG transmembrane protein fused with fluorescent and activity reporter proteins. The design was created to deliver biologically active reporters into naturally produced nanoscale exosomes by using the VSVG as an anchor. The modified vesicles were then delivered into lysosomes of recipient cells. The results were validated through multiple experiments and reported in this study.

Transfection of the vectors into HEK293 cells showed successful loading of each construct onto endosomes as demonstrated by the punctuated morphology of the images taken up to 72 hours post transfection. The morphology was tracked at three different time points and did not show any significant differences at each point. A co-transfection of designed vectors with exosomal markers, either XPACK or CD63, demonstrated that the constructs truly go into the endosomal compartment. An overlay of the RFP reporter from the designed construct with the GFP from the exosome marker was indicated by a yellow color. The data demonstrated that the constructs were localized within the endosomal compartment as MVB.

Next, we showed that the gLuc reporter protein retained its biological activity in a cell lysate in both GFP and RFP constructs. That indicated that the expression and loading process likely did not hinder the activity of the reporter. The same was observed for the puromycin resistant reporter as the cells containing the VSVG-reporter-puro constructs showed resistance to puromycin treatment.

The characterization of exosomes was done in five-fold. First, ELISA against exosome marker CD63 demonstrated that the amount of harvested exosomes per milligram of proteins for all four constructs was in the same range as the amount of harvested unmodified exosomes. That showed that exosome modification did not alter the exosome harvest yield. Next, nanoparticle tracking analysis (NTA) showed that the particle size and distribution of exosomes was in the same range as unmodified exosomes, which demonstrated that our modification did not change or vary the size distribution of the exosomes. Third, activity assay on harvested gLuc modified exosomes showed retained activity of the cargo in both the RFP and GFP constructs. That indicated that the cargo could not only be successfully loaded but it could also keep the biological activity. Confocal images of harvested exosomes were then taken to visually confirm presence of RFP and GFP reporters in exosomes. The images showed the typical punctuated morphology in all constructs. Lastly, a Western Blot confirmed the presence of exosome marker CD63 in all three tested constructs, further confirming the presence of exosomes.

In the following step, we showed that our modified exosomes were successfully taken up by three different human cell lines as well as a murine cell line. In all cases, the recipient cells started to show the presence of exosomes as soon as 6 hours post uptake assay. The data indicated both that the modified exosomes were able to be expressed and harvested, and that they could be endocytosed by recipient cells.

The goal of this work was to deliver the modified exosomes into lysosomes for a potential protein replacement therapy. Chemical staining of lysosomes after exosome uptake by two different recipient cells indicated a partial overlay of the exosomes and lysosomes. Biological staining of endosomes showed the same partial overlay of the two compartments indicating a co-localization. Uptaken exosomes become a part of endosomal pathway that can lead to either loading into lysosomes or fusion with plasma membrane,<sup>9,10</sup> which can explain the partial and not full overlay with lysosome.

After showing that the modified exosomes could be taken up by recipient cells and directed to lysosomal compartment through the endosomal pathway, we performed an activity assay on the recipient cells. A cell counting assay on the puromycin treated host cells demonstrated that the puro-modified exosomes had a slightly higher number of live cells indicating the biological activity of the cargo. For gLuc cargo, the luciferase activity increased until 48 hours post exosome uptake, demonstrating a presence of biologically active *Gaussia* luciferase.

In this work, we have engineered fused proteins that load therapeutics into exosomes. The modified exosomes can then be analyzed for activity once released into the media. Upon harvesting, the exosomes can be taken up by recipient cells and directed to a lysosomal compartment while retaining the biological activity of the cargo. For future work, we plan to exchange gLuc for a protein that replaces a missing enzyme in a specific lysosomal storage disease.

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## CONCLUSION

To address current challenges with delivery of bioactive proteins into lysosomes, we first designed vectors using VSVG transmembrane protein to deliver cargos into exosomes. We used GFP or RFP as visual reporters, and gLuc or puro as cargos. The constructs were successfully tracked in exosomes co-localized with XPACK and CD63 markers in HEK293 cells. Additionally, the producer cells showed significant biological activity. Extraction and characterization of exosomes indicated that the modification using the engineered constructs did not alter the characteristics of exosomes such as size and yield. Moreover, the engineered exosomes retained the ability to be endocytosed by recipient cells. Finally, we showed that the engineered exosomes not only co-localized with lysosomal and endosomal compartments but the loaded cargo was still biologically active in the recipient cells. The ability to engineer exosomes containing biologically active cargos allows for application in drug delivery into lysosomes.



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