Next Generation Intracellular Delivery: Optimization of Exosome Isolation and Novel Exosome -Mediated Delivery for Therapeutic Targeting of Cancer

PART 1

Optimization of Exosome Isolation through Multi Criteria Decision Analysis (MCDA)

RESEARCH SUMMARY

Research Question

- Given the current limitations in nanocarriers, can exosome isolation be optimized and exosomes be utilized as the next generation intracellular drug delivery platform for treating cancer?
- **Purpose:** Exosome isolation techniques have been optimized for utilization of exosomes as nanocarriers. This will enable the development of an efficient drug delivery platform with equal if not greater transfection rates of current nanocarriers with little to no limitations for therapeutic targeting of cancer.
- This research will significantly contribute to the fields of biomedical science, genetics, nanomedicine and the healthcare industry.
- This question can be tested through nanoparticle tracking, western blot, transmission electron microscopy, confocal imaging and utilization of Multi Criteria Decision Making Analysis for data analysis
- *This research was conducted in two parts. Part 1 - Optimization of Exosome Isolation through Multi Criteria Decision Analysis (MCDA) Part 2 - Novel use of Exosomes as Drug Delivery Nanocarriers*

HYPOTHESIS

- Exosomes, being a biological entity, have the ability to reach areas of the body that are resistant to nanoparticles and other bio-inspired systems

PART 1 PROBLEM STATEMENT

- Current exosome isolation techniques for tissue culture have not been standardized, leading to poor concentration rates, isolation times and unclean isolations. This is a barrier for utilizing exosomes as efficient nanocarriers.

OBJECTIVES

1. Identify standard exosome isolation techniques for tissue culture isolation
2. Isolate exosomes with each methodology from HEKT239 cell line
3. Accurately determine which exosome isolation technique provides cleanest and most concentrated sample of exosomes through utilization of Multi Criteria Decision Making Analysis Tool (MCDA)

BACKGROUND

Exosomes

- Exosomes are nano-sized (50-120nm) extracellular vesicles that are released by all cell types and efficiently enter other cells
- They have been shown to contain genetic material responsible from one cell to the other and mediate intracellular communication
- Current nanocarriers have many limitations that they cannot target cells selectively

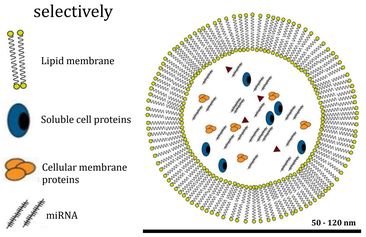


Figure 1: Components of an Exosome

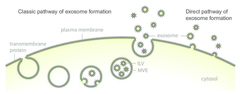


Figure 2: Formation of Exosomes

Exosomes are released upon fusion of multivesicular bodies (MVBs) with the cellular plasma membrane. They originate as intraluminal vesicles (ILVs) during the process of MVB formation

Limitations with Current Nanocarriers

- Limitations in current nanoparticle carriers include cytotoxicity as well as delivery challenges such as endosomal escape
- This occurs in three of the most commonly utilized nanocarriers: LNP's, polymer conjugates and nanocrystals

Lipid Based Nanoparticles	Delivered to the liver	
Polymer conjugates	Large amounts of conjugates necessary to achieve drug delivery	
Nanocrystals		Assemblies are not always similar to that in homogeneous solutions

Rationale for Weighted Sum Model (WSM)

- Goal: Determine most successful methodology
- Guidelines: Number of quality control tests used, all of varying importance for optimization of exosome isolation
- Necessary in Model: Importance of criteria and factoring all criteria in decision making process
- Conclusion: Weighted Sum Model is a Multi Criteria Decision Analysis tool that can be used for evaluating alternatives with relative importance

DESIGN AND METHODOLOGY

Three methodologies were selected based on research. Each individual methodology is outlined below. Cells from the ultrafiltration and ultracentrifugation methodologies were taken from the HEKT 29K3 cell line. To analyze success of methods, Weighted Sum Model (WSM) was utilized.

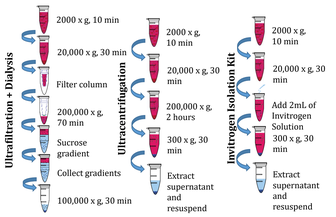


Figure 3: Exosome Isolation Procedures

Multi Criteria Decision Analysis (MCDA)

Criterion (m)	Relative Weights (wj)	Primary Parameters
Particle Size (DLS)	.25	50-120
Particle Size (Manta)	.25	50-120
PDI	.1	<.3
Concentration	.2	10^11 p/mL
Western Blot	.2	Scaled on 0 or 1 based on appearance of CD63 protein

Independent Variable: Exosome isolation technique
Dependent Variables: Particle Size, PDI, Concentration, Western Blot
Controls: Cell line, initial volume, cell growth days, centrifuge equipment

Equation used to calculate model:

$$A_i = \sum_{j=1}^{n} w_i \, a_{ij}$$

Where A_i=WSM score and a_{ij} = performance value of method

DATA COLLECTION

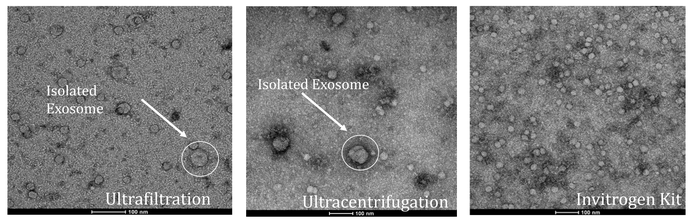
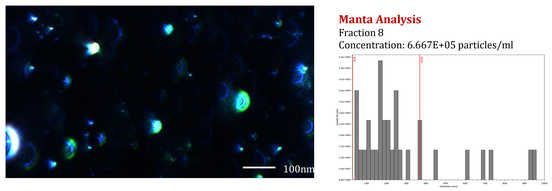


Figure 4: Transmission Electron Microscopy Imaging

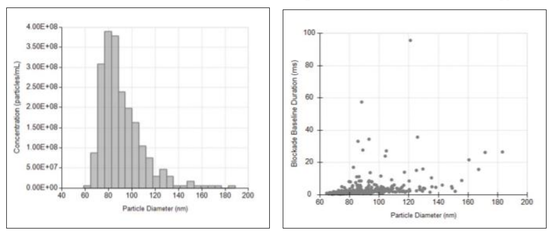
Through imaging it could be determined that Invitrogen Kit was not a viable solution for optimization of exosome isolation, as there were many other particles in the solution in addition to exosomes. Ultrafiltration and ultracentrifugation techniques were clean, and could thus be tested for quality control. As seen in figure 4, exosomes were identified in ultrafiltration and ultracentrifugation about 100 nm.

A. Quality Control Using Nanoparticle Tracking - Ultrafiltration



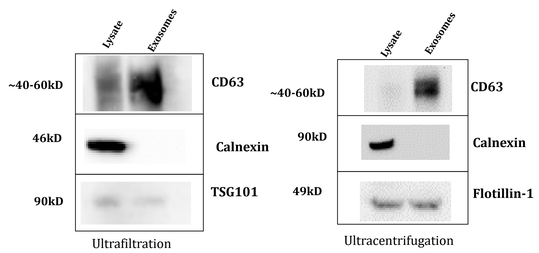
	Criterion				
	PDI	Particle Size (DLS)	Particle Size (Manta)	Concentration (p/mL)	Western Blot
Weights	0.25	0.25	0.1	0.2	0.2
Ultrafiltration	0.782	307.01	243.07	666700	1
WSM Score	**64.6**				

B. Quality Control Using Nanoparticle Tracking - Ultracentrifugation



	Criterion				
	PDI	Particle Size (DLS)	Particle Size (Manta)	Concentration (p/mL)	Western Blot
Weights	0.25	0.25	0.1	0.2	0.2
Ultracentrifugation	0.285	178	176.73	1320000	1
WSM Score	**77.9**				

c. Western Blot Protein Detection

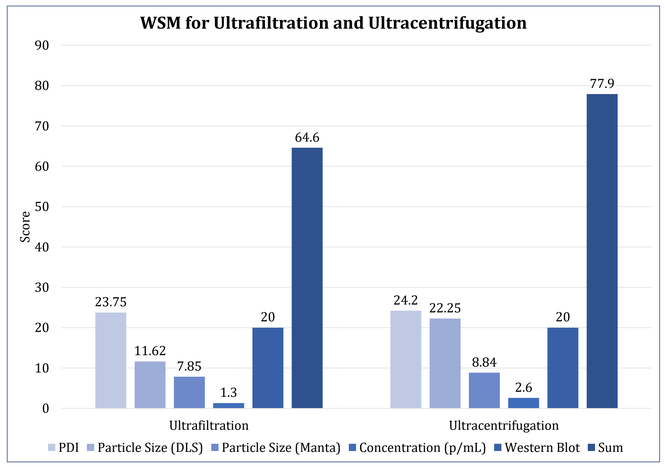


Exosome +ve markers like CD63 and TSG101 are found in both whole cell lysates and exosome lysates. Exosome –ve protein Calnexin is found exclusively in the exosome lysates.

Exosome +ve markers like CD63 is found in both whole cell lysates and exosome lysates. However, exosome –ve protein Calnexin is found exclusively in the exosome lysates.

	PDI	Particle Size (DLS)	Particle Size (Manta)	Concentration (p/mL)	Western Blot
Methodologies	0.25	0.25	0.1	0.2	0.2
Ultracentrifugation	23.75	11.62375	7.8465	1.3334	20
Ultrafiltration	24.2	22.25	8.8365	2.64	20

Weighted Sum Model



ANALYSIS AND INTERPRETATION

Visual Quality Control (Obtained through TEM)

- Invitrogen Isolation Technique is not a viable solution for exosome drug delivery, there is excess matter that cannot be considered exosomes because of their shape and size
- Most exosomes obtained from ultracentrifugation and ultrafiltration were close to 100 nm
- Utilizing the methods, there is still additional vesicles that are much smaller than 100 nm
- Some of these vesicles can include proteins and pieces of cell membrane
- This explains additional peaks found in PDI

Physical Control – Nanoparticle Tracking with Manta, DLS and Concentration

- Ultrafiltration provides few p/mL, not high enough concentration to be used
- Ultracentrifugation provides high quality exosomes, with in range Poly Dispersity Index (PDI), meaning that exosomes are relatively uniform
- Ultrafiltration PDI was too high, had a range of sizes approximating a PDI of .782
- Particle size was more within range for ultracentrifugation than ultrafiltration
- Both particle sizes from DLS and Manta showed that ultrafiltration sizes were too high, however the sizes were very different so systematic error should be accounted for in further data analysis of sizes for ultrafiltration

Biochemical Control – Western Blot

- Exosomes appear to contain CD63, a protein that is found in exosomes, for ultrafiltration and ultracentrifugation
- Calexin is not found in exosome sample meaning that sample does not contain other parts of cell from the ER, as Calexin is found in the ER
- Exosome +ve markers like CD63 and TSG101 are found in both whole cell lysates and exosome lysates.
- Exosome –ve protein Calnexin is found exclusively in the exosome lysates.
- Exosome +ve markers like CD63 is found in both whole cell lysates and exosome lysates.
- However, exosome –ve protein Calnexin is found exclusively in the exosome lysates.
- Full scores were given to ultrafiltration and ultracentrifugation because they both had clean bands for CD63, further trials should test CD9 as well

PART 1 CONCLUSIONS

1. Ultracentrifugation and ultrafiltration are standardized techniques to isolate exosomes that provide clean samples, high concentration rates and consistent isolation times

2. Ultracentrifugation is most effective technique when isolating exosomes to be used as nanocarriers because of the particle size recovered and high concentration rate.

PART 2
Novel use of Exosomes as Drug Delivery Nanocarriers

PART 2 PROBLEM STATEMENT

Exosome transfection has not yet been shown to have high efficiency comparable to nanocrystals and polymer conjugates. In addition to this, procedures to demonstrate exosome application as drug delivery platform have not been formulated.

OBJECTIVES

1. Utilize ultracentrifuged exosomes from HEK- 293T cell line
2. Create effective methodology to measure uptake of exosomes in HeLa cell line
3. Conclude that exosomes can be used as viable drug delivery nanocarriers
4. Determine uses for exosome delivery to therapeutically target cancer cells

DESIGN AND METHODOLOGY

Procedure to Stain Exosomes

Isolate Exosome
- Procedure detailed in figure 2

Run PKH26 Kit
- Add 1mL Diluent C
- Add 4uL PKH Dye
- Add 2mL 1% BSA

Washing Exosomes
- Mix and pellet at 12,000 x g for 2 hours
- Add 16mL of PBS
- Spin at 120,000 x g for 2 hours

Resuspend
- Resuspend in 1mL PBS

Imaging
- Prepare for Confocal Imaging

Independent Variable: Exosome isolation technique
Dependent Variables: Confocal imaging
Controls: Cell line, initial volume, cell growth days, centrifuge equipment

PKH26 Staining Dye
- Labels exosomes
- Utilizes aqueous solution (Diluent C) to maintain cell viability
- Fluoresces in the yellow-orange region of spectrum
- Used for monitoring uptake of exosomes

Confocal Imaging
- Optical imaging technique for increasing resolution and contrast of a micrograph
- Has the ability to identify exosomes because of its high resolution and focus

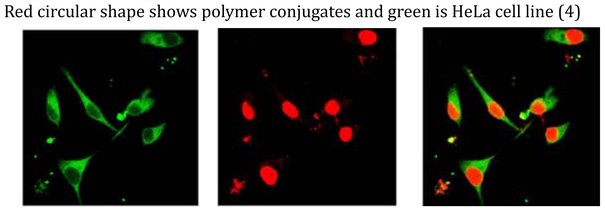
Proposed Procedure to Demonstrate Exosome Application

1. Utilize isolated exosomes and electroporate at different voltages and times
2. Introduce different concentrations of selected drug
3. Reseal exosomes by changing pH level (membrane comes together)
4. Utilize assay and count particle/mL
5. Transfect exosomes on HeLa cell line
6. Do viability reading and image after 72 hours
7. Determine if exosomes have reached cells

BACKGROUND

Polymer Conjugates

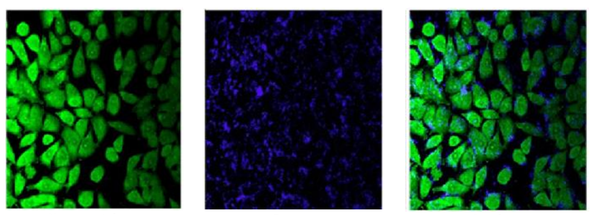
Red circular shape shows polymer conjugates and green is HeLa cell line (4)



Polymer Conjugates | Drug | Overlap

Nanocrystals

Green is nanocrystals, blue is introduction of the drug (5)



Nanocrystals | Drug | Overlap

Goals

1. **Demonstrate that exosomes can efficiently be taken in by cell line**
2. **Compare this efficiency to that of nanocrystals and polymer conjugates**

DATA COLLECTION - CONFOCAL IMAGES

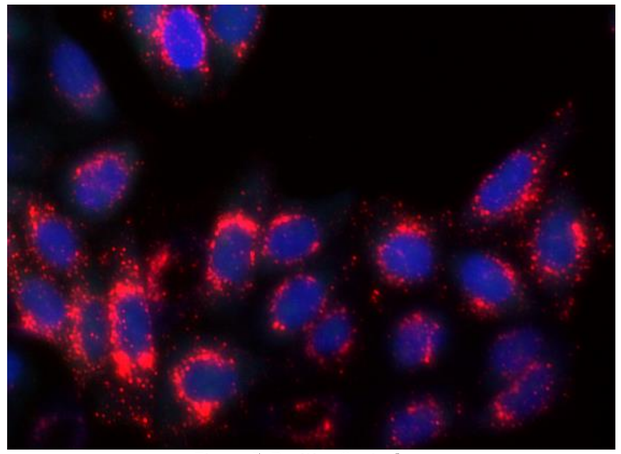


Figure 5: Positive Control

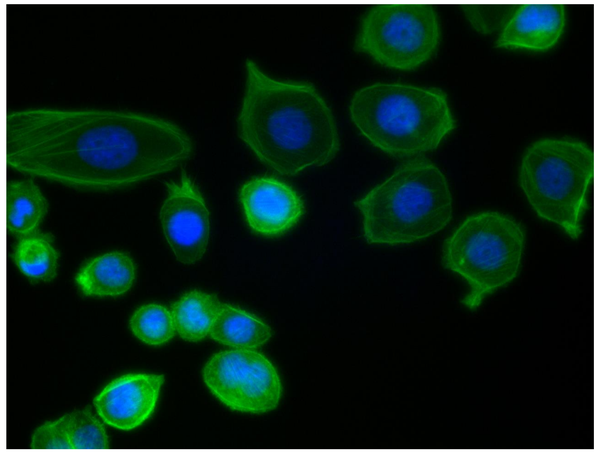
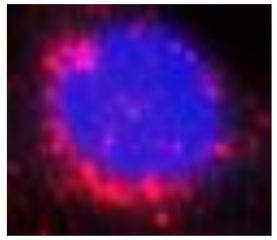


Figure 6: HeLa Cells Negative Control

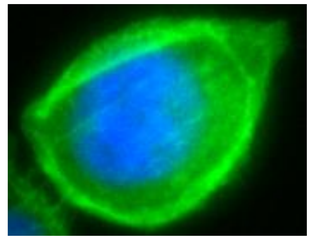
ANALYSIS AND INTERPRETATION

Observations - Positive Control	Observations - Negative Control
• Blue circular shapes in control represent nuclei • HeLa derived cell line • Pink nanoparticles stained with PKH26 Dye	• Green outlines cell • Blue circles represent nuclei • Cell line without introduction of exosomes
• HeLa cell line • Benefits of utilizing HeLa cell line: HeLa cell line is cancer derived and grows rapidly	
• Demonstrates that exosomes can be isolated from one cell line and introduced into a different cell line at large transfection rates	
• Drug delivery systems such as mRNA need to enter cytoplasm to become effective, these images show that the nanoparticles have reached close to the nucleus thus proving effective.	
• Multiple particles enter one cell at a time, ensuring that drug delivery will be efficient with exosomes	
• Average amount of exosomes per cell is relatively equal, analyzing specific pattern of uptake can be beneficial to exosome drug delivery platform	

Analysis of Cells



Exosomes and Nucleus in HeLa cell



HeLa cell and Nucleus

PART 2 CONCLUSIONS

Cells introduced to HEKT-239 cell line
- Confocal imaging showed that exosomes have high transfection rates

Transfection compared to negative control
- Transfection rates are comparable to nanocrystals and polymer conjugates using confocal imaging comparisons

Drug Delivery Exosome Procedure
- Exosome drug delivery procedure was formulated to demonstrate application of exosomes as drug delivery platform

RESEARCH CONCLUSIONS

PART 1: Optimization of Exosome Isolation through Multi Criteria Decision Analysis (MCDA)

1. All techniques identified (ultracentrifugation, ultrafiltration and Invitrogen isolation kit) can isolate exosomes
2. Through MCDA tool it was determined that **ultracentrifugation** was the most successful technique for optimization of exosome isolation

PART 2: Novel use of Exosomes as Drug Delivery Nanocarriers

1. Exosomes have the ability to enter cells and can be observed from confocal images that will resemble positive control as seen in figure 5
2. **Exosomes can be used as a novel platform for drug delivery and therapeutic targeting of cancer**

FUTURE DIRECTIONS

A. LNP Encapsulated Exosome

- Lipid Based Nanoparticles (LNP's) are limited to there delivery to the liver
- A combination of an LNP and an exosome could result in an increased uptake percentage as well as a ability to go targeted areas of the body
- These combined platforms could result in an effective drug delivery technique that would be accepted by all cells
- Because LNP's are larger than exosomes, this delivery system would also be able to hold larger genetic materials and technologies such as CRISPR/Cas9

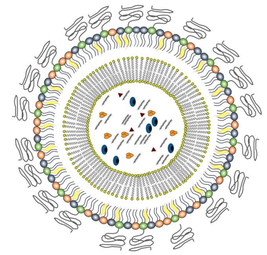


Figure 6: LNP Encapsulated Exosome

B. mRNA Encapsulated Exosome

- A leading genetic technology in the field drug therapeutics is *in vitro* transcribed (IVT) mRNA
- IVT mRNA has multiple benefits over conventional DNA plasmids and short interfering RNA's
- IVT mRNA does not need to enter the nucleus to be functional, only the cytoplasm
- Additionally, IVT mRNA is transient, meaning that it does not last more than 24 hours and it does not integrate into the genome, preventing the risk of insertional mutagenesis

C. Electroporated Exosome

- Electroporation is a process through which high voltage shocks are induced in attempts to permeate a membrane.
- This process is often used to permeate cell membranes and show a high efficiency
- Electroporation may also be able to permeate an exosome membrane long enough for siRNA to be loaded into the exosome
- This could also prove a solution for encapsulating mRNA in exosomes

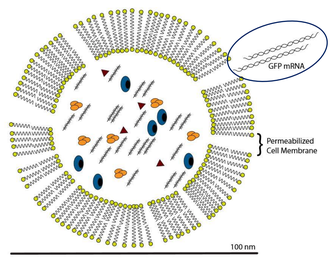


Figure 7: Electroporated Exosome

Major References

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All figures made by finalist