High-Throughput Screening of Engineered Exosomes for Expedited Therapeutic Development

Introduction: Development of personalized medical treatments and diagnostics is limited by our ability to engineer tools that can keep up with demand. Exosomes are a type of nano-sized extracellular vesicle (EV) naturally produced by cells and released via endosomal fusion with the plasma membrane. Although primarily utilized in the detection of diseases such as cancer, exosomes are increasingly being investigated as a means of drug delivery due to their potential for multi-functional targeting and inherent biocompatibility [1]. A longstanding setback in the study and application of exosomes for therapeutic purposes is the lack of standardized methods with which to isolate, concentrate, and characterize them [2]. Although scientists can functionalize exosomes with targeting molecules and drug payloads, scale-up is limited by the ability to quickly identify favorable processing conditions and thus good manufacturing practices. **In this project, I propose creating a system that will reduce the burden of the screening process by developing a tool to rapidly assess exosome production and functionality.** This study will help others engineer EVs as a tool for medical and non-medical applications.

Traditional methods quantify exosomes via nanoparticle tracking analysis (NTA) which relies on light scattering of particles to judge size and concentration via analysis of Brownian motion. However, NTA also counts non-exosome particles such as protein aggregates, leading to large discrepancies in actual determination of exosome quantity in solution. Moreover, NTA does not account for functionality of engineered exosomes, which typically display targeting molecules on their surfaces. Current exosome purification methods are labor intensive, requiring multiple days of centrifugation and gradient separation to remove the crude EV material prior to quantification and analysis of the exosome product [2]. Therefore, developing a method to rapidly quantify and assess exosome functionality with targeting molecules would enable scientists to focus their attention on scaling up and purifying only the most promising therapeutic exosome processes. This development would bypass the current time-consuming roadblocks associated with engineered exosome production, advancing the field.

Objective: I will establish a high-throughput method to screen processing conditions for engineered exosomes. Protein microarrays, which have previously been used in diagnostic exosome assays [3], have the potential to quantify functionalized exosomes in an efficient and accurate manner. Therefore, *I hypothesize that protein microarrays can be utilized as a high-throughput method to screen processing conditions for engineered exosomes*. I plan to (1) investigate optimal microarray conditions by engineering spot formulations and process steps and (2) evaluate ideal processing specifications for exosome production by running protein microarrays in tandem with cholesterol-based quantification standards.

Aim 1: Investigate optimal microarray conditions to create a high-throughput screening tool for functionalized exosomes. Protein microarrays can be modified to include different antibodies in each spot. The ideal formulation would be one that binds only targeted exosomes. Exosomes express the surface ligands CD9, CD63, and CD81 as unique identifiers from other EVs [2,3]. Antibody cocktails for these ligands, as well as the expressed targeting molecules, would enable binding of only the desired exosomes—even in the presence of a crude EV sample—while washing away all other materials in solution. I will determine the concentration of each antibody for optimal exosome binding kinetics, which is a function of the desired targeting molecule antibody. The formulation must also be modified to include a solvent material that is suitable for microarray printing but that does not interfere with the antibody-exosome interactions. Once I find the ideal antibody cocktail ratios, I will test the formulation to ensure that it prints appropriately on the microarray slides. The solution's fluid properties must enable it to flow easily for spotting on the slides, dry quickly, and spread evenly. Different additives noted in literature would be investigated to find those that work best with the solution. I expect that a 5% glycerol content will produce the desired results, as it was used in a previous publication illustrating a method

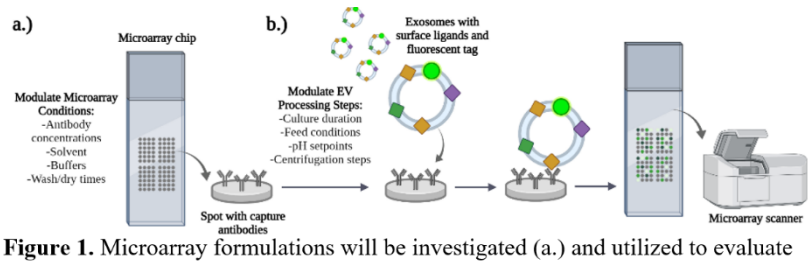

Figure 1. Microarray formulations will be investigated (a.) and utilized to evaluate EV processing steps (b). Created with BioRender.com.

for exosome phenotyping using protein microarrays [3]. Various factors affect microarray accuracy, including sample application times, wash and blocking buffer identities and concentrations, number and duration of washes, and drying times. Each of these factors will be explored systematically using a factorial design of experiments (**Fig.1a**). If the microarray cannot be optimized for EVs, a column-based affinity separation method could be used to purify and confirm functionalization of exosomes. Completion of this aim will optimize the microarray as a tool for engineered exosome analysis.

Aim 2: Evaluate ideal process conditions for engineered exosome production. Having identified the most favorable microarray conditions, I will compare processing conditions for engineered exosomes to prove the rigor of the quantification method. With this high-throughput method, dozens of different processing specifications—such as days in culture, feed conditions, pH setpoints, and centrifugation steps—could be analyzed simultaneously with a minimal demand on sample volume. The first step in engineering a suitable high-throughput method is to create a reliable standard for comparison. While working at Codiak Biosciences, I pioneered a cholesterol assay that circumvented time-consuming NTA by quantifying exosomes based on their cholesterol content and presented a poster on this work at the International Society for Extracellular Vesicles (ISEV) 2020 Annual Meeting. The fluorescence plate-based Amplex Red Cholesterol Assay was quick and accurate within a prescribed range of 0-8 µg/mL. Employing it also enabled me to quantify exosomes without the need for purification, reducing cost and time spent. I will utilize this preliminary work to develop a standard method for exosome quantification for this project. Once the standard is established, exosome samples conjugated with fluorescent tags will be applied to the microarray spots. After washing, only the exosomes with the desired surface ligands will bind to the spot and be fluorometrically detected (**Fig.1b**). The relative fluorescence of each spot will be compared to a standard where only the exosome detecting antibodies, not the targeting molecule antibody, are present. This relative fluorescence describes the number of functionalized exosomes in the sample. These signals will be compared to the cholesterol-based assay to obtain a quantitative readout of the number of engineered exosomes and their relative protein loading. If fluorescent tags are not feasible, dyes, luminescent substrates, or other types of markers could be conjugated to the exosomes to create a measurable readout. The results of this aim will elucidate the ideal conditions for engineered exosome screening.

Intellectual Merit: This work will generate a deeper understanding of protein microarrays as high-throughput screening tools and can be applied to development of new exosome or nanoparticle-based technologies, which could be used in applications ranging from drug delivery to water treatment. It will expand the foundational knowledge surrounding EVs and support future endeavors to optimize the production of different types of engineered exosomes, aiding in the discovery and development of EV therapies. As a member of the Leonard Lab, which has expertise in exosome production and engineering, I am well positioned to complete this project.

Broader Impacts: This proposal integrates chemical engineering, bioengineering, and biochemistry principles to create an interdisciplinary project that advances dynamic drug delivery platforms through high-throughput screening. Successful completion of this project will enable accurate exosome quantification, reducing labor and time investments. Rapid identification of improved processing conditions will support efficient and sustainable production of therapeutic exosomes, increasing manufacturing feasibility, and thus increasing their eventual accessibility on a global scale. I will leverage my connections at Codiak Biosciences to establish a collaboration to facilitate and support the project. I will disseminate the knowledge gained from this research to the scientific community for feedback and further development through conferences and publications, such as the ISEV Annual Meeting. Providing students with opportunities to learn about STEM fields and to participate in projects directly will foster the next generation of research scientists. I plan to direct my outreach toward programs encouraging underrepresented students to consider graduate school, such as REUs and the Northwestern Morning Mentors and Mentorship Opportunities for Research Engagement (MORE) programs, where I will mentor students and encourage their participation in STEM research.

References: **[1]** Wang, J. et al. (2017). *ACS Applied Materials & Interfaces, 9*(33), 27441-27452. **[2]** Chia, B. S. et al. (2017). *TrAC Trends in Analytical Chemistry*, 86, 93-106. **[3]** Jørgensen, M. et al. (2013). *Journal of Extracellular Vesicles*, 2(1), 20920.