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ExoSense: A novel technology for selective purification of CD63+ exosomes from neural cells

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EXOSENSE: A NOVEL TECHNOLOGY FOR SELECTIVE PURIFICATION OF CD63+ EXOSOMES FROM NEURAL CELLS

by

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ABSTRACT

Exosomes are extracellular, cell signaling microvesicles that contain unique genomic and proteomic signatures reflective of the host cell's pathophysiological conditions. In recent years, the study of exosomes has increased tremendously because many have been recognized as molecular biomarkers with the potential to advance methods of disease diagnostics and therapeutics as well as contribute to physiological analyses of multiple organism types. With the promising potential that exosomes offer to the field of molecular biology, it is vital to establish an efficient and consistently reliable mechanism of exosome isolation from biological samples. Many isolation techniques currently available typically yield exosome samples with numerous contaminants, making them low in purity for exosome content. Our lab has developed a novel technology for solid-phase exosome purification directly from biological samples. This study utilizes SDS-PAGE and fluorescent imaging analysis to assess the specificity of the ExoSense microprobe-based exosome isolation technology. The proteomic profile generated from the SDS-PAGE shows fewer bands with a cleaner background for the microprobe-based sample compared to the traditional polymer precipitated exosome proteins, suggesting an exosome population higher in purity from the microprobes than what the polymer reagent provides. Fluorescent imaging resulted in distinct differences between the control and exosome-specific groups, indicating an exosome-specific population captured using the probes. Results from this study can be used collectively to

validate the specificity of the novel ExoSense exosome capture technology. Future studies can be applied for optimizing the technology for commercial applications.

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DEDICATION

This work is dedicated to my mom who inspired my passion for science, to my dad who taught me the value of hard work and to trust my instincts, to my brothers who encouraged me to never give up, and to my incredible husband who has supported me through everything.

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CHAPTER 1

INTRODUCTION

1.1 Objective

The goal of this study is to validate the specificity of a microprobe-based method for direct immunocapture of a CD63-positive subpopulation of exosomes via SDS-PAGE and fluorescent imaging. We hypothesize that the microprobes are capable of selectively isolating a subpopulation of exosomes carrying specific surface protein markers. CD63 is a member of the tetraspanin family that is one of the most abundant proteins found on the surface of exosomes and thereby is expected to provide selectivity to the isolation procedure (Pols and Klumperman, 2009). Biotinylated stainless-steel microprobes (130 μm in diameter, 30 mm in length) will be functionalized with an anti-CD63 antibody conjugated to streptavidin that selectively binds to this protein marker expressed by the exosomes. The probes will be used for selective capture of extracellular vesicles directly from astrocyte-conditioned cell media. Our preliminary results indicate that the average loading capacity is 2.8×10^6 exosomes per probe. We aim to confirm our hypothesis with two experimental objectives: 1) analyze the proteomic content of captured vesicles using SDS-PAGE analysis, and 2) characterize the captured vesicles on the surface of the probes using fluorescent imaging analysis.

1.2 Rationale

Exosomes are extracellular vesicles that are indicative of the pathophysiological conditions of the host cell and can modify the physiological response by reprograming the recipient cell (Valadi et al., 2007). While exosomes are released under normal physiological conditions, their number is increased upon neurodegeneration or neoplastic transformation and are emerging as crucial biomarkers for disease diagnostics (Soung et al., 2017). Astrocytes regulate brain function by maintaining ion, metabolic, and neurochemical homeostasis. The nature of astrocyte-to-neuron communication is mediated by direct cell-to-cell contact as well as by a complex array of exosomes that carry a diverse pool of non-coding microRNAs capable of reprogramming protein expression in recipient cells. Therefore, astrocyte-derived exosomes from these cells give insight into the physiological status of the nervous system (Men et al., 2019). Selective isolation and genetic analysis of exosome subpopulations that express the same surface protein markers is crucial in studies of the central nervous system (Kowal et al., 2016).

1.3 Significance

The proposed exosome purification technology is based on a microprobe method for site-specific exosome purification that is non-destructive to the biological specimen. Selective isolation of exosome subpopulations that express the same surface protein markers is crucial in proteomics and genomics studies of extracellular vesicles (Kowal et al., 2016). Because of their distinct surface proteins and vesicular content, exosomes have the potential to have a significant impact on molecular biology studies. For example, CD63 positive exosomes are present at a higher level in malignant cells compared to normal cells (Logazzi et al., 2009). However, the potential use of exosomes in a clinical

or research setting is fully dependent on the successful isolation of exosomes from biological samples. The developed ExoSense microprobe-based technology is expected to have an important impact in the field of extracellular vesicle research as the immunocapture approach will enable selective purification of exosomes expressing the same surface marker and enable integration with standard systems for genomic and proteomic downstream analysis.

CHAPTER 2

BACKGROUND

2.1 Exosome Structure and Biogenesis

Exosomes are small extracellular cell signaling molecules that have been increasingly studied in the last decade as their structural features and physiology provide evidence of these vesicles playing a large role in various cell signaling and homeostatic regulatory processes (Mathieu et al., 2019). The earliest studies of exosomes focused on characterizing the inner structural components which differentiate exosomes from other microvesicles. Valadi et al. performed an extensive genetic analysis on exosomes and compared the results with a genetic analysis of the host cells because they hypothesized that exosomes contain unique messenger RNA (mRNA) and microRNAs (miRNA) which are involved in post-translational alterations in the recipient cell. These studies confirmed that exosomes contain specific miRNAs and mRNAs which can alter the biological activity of the recipient cell post-translationally. The results of this study provided evidence of exosomes being potentially critical regulators of cellular homeostasis (Valadi et al., 2007).

Exosomes are extracellular vesicles that are endosomal in origin and actively mediate communication between cells. The intracellular pathway of exosome biogenesis begins with the formation of endosomes from an enfolding cellular membrane that contains varying proteins and other signaling molecules. This early endosome is

processed in a membranous system referred to as multivesicular bodies (MVB) (Hessvik & Llorente 2018). Within the MVB compartments, the cell-signaling molecules are sorted into new vesicles for subsequent release from the cell. New molecules are also oriented into these spaces depending on the directed needs of the signaling molecules. This stage of exosomal development is orchestrated by four multiprotein complexes referred to as the endosomal sorting complexes required for the transport (ESCRT) pathway (Figure 2.1). There is also a pathway that directs the formation of exosomes independently from the ESCRT proteins. However, this pathway is less well defined (Ha et al., 2016).

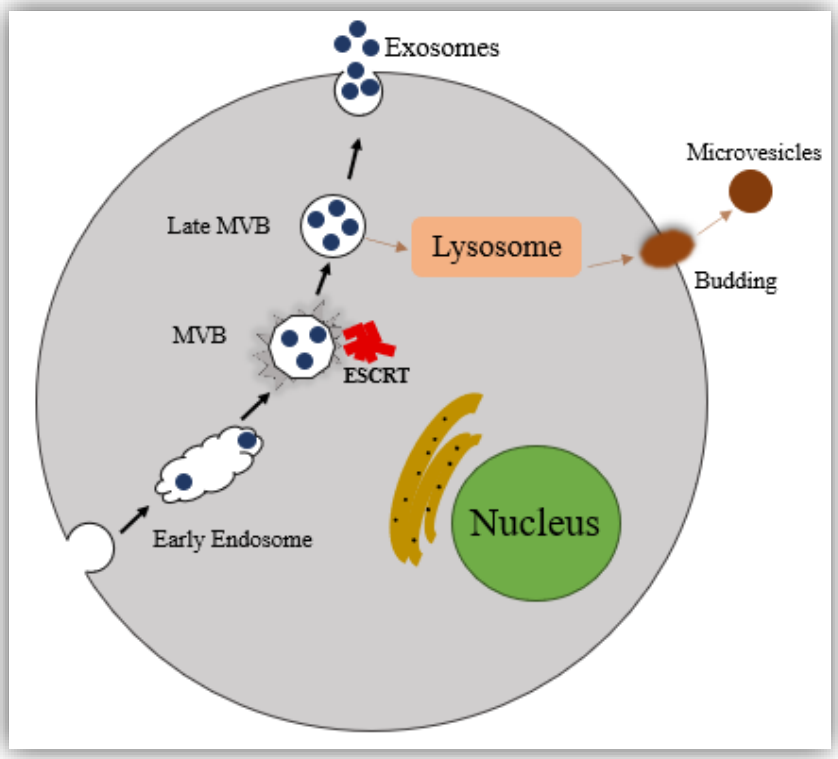


Figure 2.1: Exosome vs. microvesicle biogenesis pathway. Overview of exosome formation via endocytosis, development into multivesicular bodies, the orientation of new molecules mediated by ESCRT, and late multivesicular body formation. Microvesicles form by budding orchestrated by lysosomal processing of late MVBs. Exosomes are excreted via exocytosis of late MVBs. *Figure adapted from Ha et. al, 2016 Figure 2.*

2.2 Exosomes in Biomedical Research and Diagnostics

Exosomes are signaling vesicles that have recently emerged as biomarkers and a drug delivery platform for the advancement of disease diagnostics, monitoring, and therapeutics. Exosomes reflect the pathophysiological conditions of the host cell and can modify the physiological response by reprogramming the recipient cell (Mathieu et al. 2019). Exosomes contain unique protein markers on their surface as well as proteins, miRNAs, and mRNA of their cell of origin that allow them to be used as disease biomarkers (Valadi et al. 2007). Exosomes mediate immune responses (Anel et al. 2019),

cancer progression (Soung et al. 2017), and central-nervous system-related diseases (Liu et al. 2019).

In cancer diagnostics, several specific exosomal biomarkers have been identified through a comprehensive analysis of cancer cell-derived exosomes. For example, CD24 and EpCAM are two exosomal proteins associated with both breast and ovarian cancer, while EGFR seems to be unique to glioblastomas (Soung et al., 2017). In Parkinson's studies, exosomes were found to indirectly upregulate the expression of Syntenin, which now serves as a specific biomarker for tissue changes in the onset of neurodegenerative disease (Tomlinson et al., 2015). Both of these studies identify specific exosomal properties as unique biomarkers in complex diseases, vastly supporting the potential of exosomes to contribute advancements in biomedical disease diagnostics and therapeutics.

Exosomal membranes are enriched in tetraspanins (CD63, CD9, and CD81) that are used as biomarkers for disease diagnostics and prediction of therapeutic response (Andreu and Yanez-Mo 2014). Isolation and genetic analysis of antigen-specific vesicles from the total exosome population are crucial for understanding the state of degenerating tissues that are otherwise inaccessible in the central nervous system (Kowal et al. 2016). Astrocyte-neuron communication is the key regulator of overall brain function, so exosomes from these cells serve as important indicators of the progression of neurological diseases in brain tissues (Men et al. 2019). There is no current method that noninvasively isolates exosome subpopulations, which all share membrane-specific tetraspanin markers, from other vesicles. Isolation of a pure exosomal population from biological tissues is an important prerequisite for the development of extracellular

vesicle-based therapies and the discovery of disease-specific biomarkers (Kowal et al. 2016).

2.3 Overview of Exosome Purification Techniques

The current methods of exosome isolation all have limitations that prevent the application of exosomes in a clinical or laboratory setting, and therefore prevent the study of exosomes from reaching their full potential. Multiple technologies that improve exosomal isolation have been developed, and the process has rapidly evolved in recent years. Methods of exosome isolation currently include ultracentrifugation and filtration, size-exclusion techniques, polymer precipitation, immunoaffinity, and microfluidics (Doyle and Wang 2019). Most ultracentrifugation methods are coupled with filtration to increase total exosomal yield. This method can vary slightly from protocol to protocol but includes a series of ultracentrifugation steps at high speeds with long assay times. Ultracentrifugation generally has a low exosomal yield (< 5%), meaning that less than 5% of the total sample volume recovered in the final product consists of microvesicles and high rates of contamination due to tearing of the membranes and clumping (Livshits et al. 2015). A size-exclusion approach yields a purer exosome subpopulation compared to ultracentrifugation because microvesicles are very small in size and the solid phase of the chromatography column removes cell lysate or other sources of contamination (Gheinani et al. 2018). Although size-exclusion has a simpler protocol and involves more readily available equipment, it still has a low total yield (< 8%) and provides a population of exosomes that express a variety of surface markers (Doyle and Wang 2019).

Polymer precipitation is the most common approach to exosome isolation. It requires little equipment, encompasses a simple protocol, and provides a relatively high

total product yield (≥ 25%). This method, however, can only process a very small sample volume, has a long assay time (8 h or more), and carries the risk of compromising the structural integrity of the microvesicles isolated (Konoshenko et al. 2018). Immunoaffinity techniques largely involve the use of antibody-coated beads which are target-specific and can be used in almost any biological media. This method is highly specific and has a high total yield and purity at the time of capture (> 99%). However, the reagents required to remove the exosomes from the beads for analysis destroy most of the microvesicle product, rendering them inadequate for scientific analysis (Doyle and Wang 2019).

Microfluidics strategies attempt to combine lab-on-a-chip technologies with common isolation methods including size-exclusion (Lee et al. 2015) or immunoaffinity (Zarovni et al. 2015) for exosome isolation. The main goals of these techniques are target-specific capture while reducing potential contamination from excess cell debris or buffers and increasing the overall total product yield. These microfluid methods, however, have no standardized protocol and yield a variety of experimental results (Yu et al. 2018). The advantages and disadvantages of current exosome capture methods can be summarized by comparing the most common exosomal isolation techniques (Table 2.1).

Table 2.1: Comparison of current exosome isolation methods. Product yield is the percent of total sample volume recovered in the final product which consists of microvesicles. The concentration is indicated by the number of particles per mL (ppm) that are measured in the final product, determined by nanoparticle tracking analysis. Sample volume is how much volume can be processed by the assay. Primary advantages and disadvantages are listed.

Method	Ultracentrifugation + Filtration	Polymer Precipitation	Immunoaffinity Beads	Microfluidics
Product Yield	5-25%	$\geq$ 25%	99%	42-97%
Concentration (ppm)	1.1×10^9	1.8×10^{11}	N/A	High; (amount unspecified)
Sample Volume	> 100 mL	≈100 µL	< 1mL	20-50 µL
Primary Advantage	Processes large sample volume	Simple protocol	Target specific, yielding high purity	Highest recorded product yield and purity level
Primary Disadvantage	Lowest recorded product yield and purity level	Low purity level, damaged vesicles	Unable to characterize recovered vesicles (from damage)	Unstandardized protocol, leading to varied experimental results

2.4 ExoSense Solid-Phase Purification of Extracellular Vesicles

The exosome purification technology that we are developing is based on a microprobe method for noninvasive and site-specific exosome purification and genetic analysis. Stainless-steel exosome capture pins (130 µm×30 mm) are functionalized with an anti-CD63 antibody that selectively binds to the corresponding tetraspanin (Figure 2.2). A microprobe-based approach to exosome isolation eliminates the need for large, expensive equipment in the protocol and creates a platform that can elegantly interface with downstream genomics and proteomics technologies for point-of-care diagnostics. . Microneedles are non-destructive to the sample and are therefore a preferred method of

laboratory testing in a medical setting. This developed approach could reduce the current lengthy, multistep exosomes isolation procedures to a single step.

To produce site-specific exosome capture directly from biological samples, a stainless-steel microprobe was functionalized with an antibody specific to a tetraspanin (CD63) that is expressed on the surface of the vesicles. The first layer coating the needles consists of alternating oppositely charged polyelectrolytes via layer-by-layer assembly. Five bilayers of positively charged polyethylene and negatively charged polyacrylic acid were adhered first to produce a stable precursor layer for the covalent linkage of biotin followed by the immobilization of streptavidin-conjugated anti-CD63 antibodies. Carbodiimide cross-linker chemistry (EDC) is used to form the chemical bond between the amine group of the biotin and the carboxyl group of the polyacrylic acid (Lvov et al. 1995). Streptavidin-conjugated anti-CD63 forms a covalent bond with the biotin, providing stable immobilization of the anti-CD63 antibody on the surface of the probe. This integration of this immobilization technique on the surface of the microprobe enables the development of a non-invasive tool for the immunoisolation of exosomes directly from biological sources without the requirement of an intermediate, exosomes pre-enrichment step.

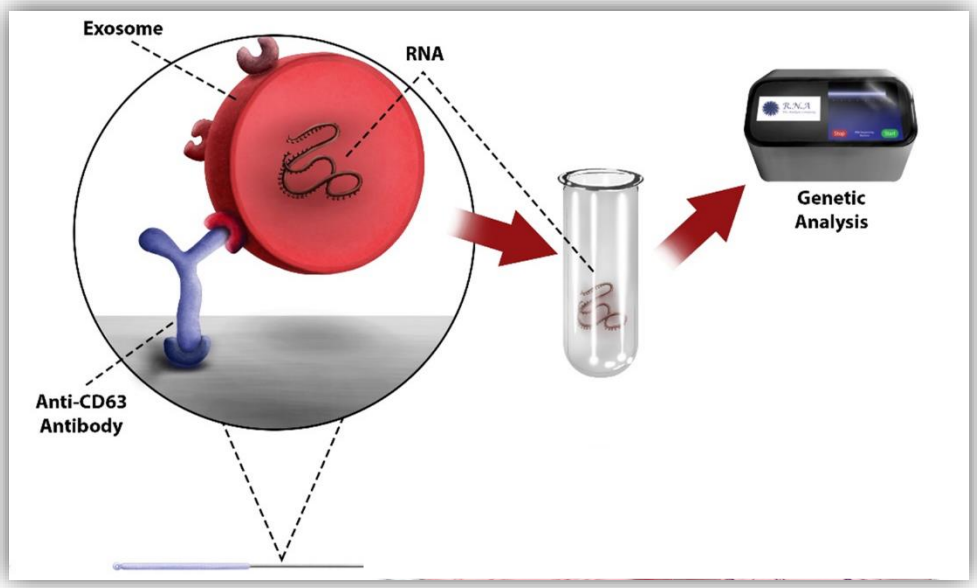


Figure 2.2: Schematic of the anti-CD63 functionalized microprobe as a sensitive bioanalysis platform for purification of exosomes.

2.5 Proteomic Analysis of Extracellular Vesicles

2.5.1 SDS-PAGE

Electrophoresis is a technique that separates proteins based on their size in an electric field gradient. A mixture of protein samples moves from the positive electrode to the negative one. Since proteins with lower molecular weight migrate faster, the mixture is separated based on the size of the peptides. Proteins have a variable charge that is determined by the type and sequence of the amino acids. To impart an overall negative charge, the proteins are coated with sodium dodecyl sulfate (SDS) before running the gel (Manns, 2011).

SDS-PAGE is a useful tool for exosome studies because the protein profile of collected samples can be visually compared. In one study, SDS-PAGE was used to determine if protein bands were thicker in exosomes isolated from plasma versus serum-extracted exosome samples. From these SDS-PAGE results, the authors were able to suggest which of the two biological sources of exosomes may yield a stronger proteomic signal (Grunt et al., 2020). Another study uses multiple SDS-PAGE analyses as supporting evidence that exosomes collected from the same biological source at different time points contain an unpredictable number of proteins (Burkova et al., 2019).

Our study utilizes SDS-PAGE analysis to assess the differences and similarities in the protein bands profile among total cellular protein samples, exosomes purified via traditional polymer precipitation, and exosomes purified via the ExoSense capture pin technology. SDS-PAGE is useful to our study because we can compare how the protein content of both exosome samples compares with the protein purified from the total cell lysate. Applying SDS-PAGE to this study will help us assess the specificity of the

ExoSense capture technique and to predict the identity of the protein bands within the gel.

2.5.2 Western Blot

Western Blot is a laboratory technique that is commonly used for the detection of a specific protein in a variety of biological sample types. It is both a size exclusion and an antigen-specific method of protein detection that allows for accurate quantification of the protein of interest. The method is also used for the detections of posttranslational modifications such as phosphorylations and glycosylation in biological specimens (Kurien & Scofield, 2006).

Because Western Blot enables highly specific protein detection and quantification, it is also used to assess both the proteomic content of exosomes and to validate the efficiency of the extraction method. For instance, one study used Western blot to confirm the presence of specific protein markers that distinguish between vesicle subtypes in a biological sample (Kowal et al., 2016). Another study utilizes Western blot to characterize TNF-α-like exosomes in the immune system as biomarkers for arthritis (Zhang et al., 2006). In these studies, however, Western blot is used in addition to another method of confirmation such as mass spectrometry for validating exosome protein markers because other vesicle subtypes are often present and may interfere with the antibody signal in the isolated exosome samples (Dash et al., 2021). Applying Western blot to exosome characterization studies is a useful tool for validation of the exosomal protein markers amongst other vesicle subtypes (Kowal et al., 2016).

2.6 Fluorescent Imaging Technique

Fluorescent immunodetection is a molecular biology technique that can be used to identify and characterize distinct features on the surface of cells or other biological samples using a fluorophore-conjugated antibody. At the core of this approach is a molecule that absorbs light or other electromagnetic radiation and emits light in a longer wavelength frequency (Hildonen et al., 2016).

Fluorescent imaging can be used to assess the efficiency of the ExoSense probe enrichment via detection with tetraspanin-specific primary antibodies followed by a fluorophore-conjugated secondary IgG antibody. The presence of exosome-specific surface markers will be assessed using primary antibodies against native epitopes of CD9 and CD81 therefore visually characterizing the tetraspanin surface proteins of the captured exosomes. The fluorescent approach for characterizing and imaging exosomes can be performed on the surface of the microprobes without removing the exosomes from the probe or lysis of the vesicles (Figure 2.3).

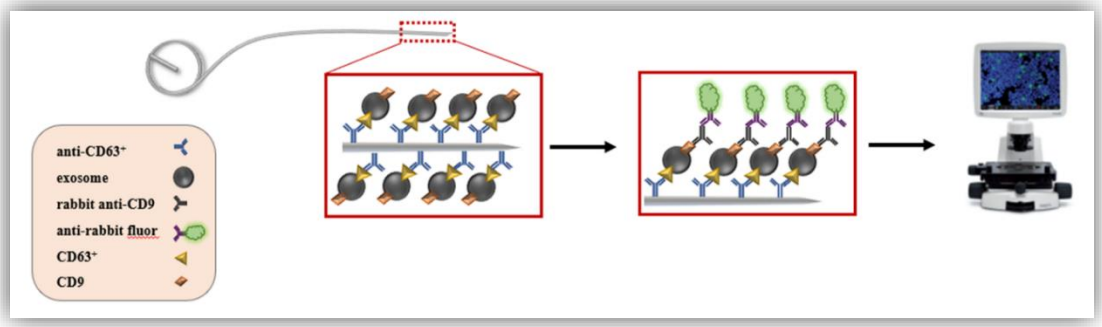


Figure 2.3: Schematic of exosome isolation and fluorescence imaging. Exosomes will adhere to the surface of the microprobes through antibody-specific interactions of anti-$CD63^+$ (biotinylated to needle surface) and $CD63^+$ (exosome surface protein). Exosome morphology can be characterized with imaging of the CD9, anti-rabbit fluorophore antibody system.

CHAPTER 3

METHODS

3.1 Layer-by-layer Functionalization

At the core of the ExoSense solid-phase exosome purification technology were stainless steel probes (130 µm×30 mm) functionalized with a layer-by-layer (LbL) assembly which consists of 6 bilayers of polyelectrolytes with opposite charges. Before LbL immobilization, the probes were polished with sandpaper. To remove organic materials, the probes were ultrasonically washed in acetone for 5 minutes, ultrapure water for 5 minutes, in hexane for 15 minutes, and then in ultrapure water heated to 50 °C for 15 minutes. The probes were then ultrasonically washed in acetone again for 5 minutes and dipped in acetone heated to 50 °C for 15 minutes. To create a uniform hydroxyl group coverage, the probes were etched using sulfochromic acid (prepared by dissolving 6 g of $K_2Cr_2O_7$ in 100 mL of H_2SO_4) at 60 °C for 10 minutes and stored under a vacuum until LbL assembly.

The etching step lays a solid foundation on the probes for the polyelectrolyte immobilization by coating them with charged OH molecules that will strongly adhere the first polyelectrolyte layer to the surface of the probes. The first polyelectrolyte layer consists of negatively charged poly(ethylenimine) (PEI) (MP Biomedicals, Cat. # 195444) and was prepared by dissolving 6 mL of PEI in 94 mL of D.I. water along with 25 µL of 0.5 M NaCl to obtain a 3 mg/mL PEI solution with a pH of 5. HCl and NaOH

were used to adjust the pH to the required value for the polyelectrolyte solutions. The second polyelectrolyte layer consists of positively charged poly(sodium 4-styrenesulfonate) (PSS) (Sigma-Aldrich, Cat. # 243051) and was prepared by dissolving 60 mg of PSS in 20 mL of D.I. water along with 25 µL of 0.5 M NaCl to obtain a 3 mg/mL PSS solution with a pH of 8. All remaining positively charged layers were orchestrated using polyacrylic acid (PAA) (Sigma-Aldrich, Cat. # 523925), and PAA was prepared by dissolving 6 mL of PAA in 63.6 mL of D.I. water and 25 µL of 0.5 M NaCl to obtain a 3 mg/mL PAA solution with a pH of 8.

The layer-by-layer assembly was immobilized with alternating charged polyelectrolyte layers. The probes were dipped in each solution for their allotted time, placed in D.I water for rinsing, and allowed to air dry before adding the next layer: PEI – 45 mins, PSS – 15 mins, PEI – 15 mins, PAA – 15 mins, PEI – 15 mins, PAA – Overnight (16 hours), PEI – 1 hour, PAA – 30 mins, PEI – 30 mins, PAA – 30 mins, PEI – 2 hours, PAA – 30 mins. Following the layer-by-layer assembly, all probes were immobilized with a biotin-EDC linker complex to provide a stable adherence of the primary antibody for exosome capture. The biotin (ThermoFisher, Cat. # 21346) was prepared at 50 mM and the EDC (ThermoFisher, Cat. # 22980) solution was prepared at 100 mM. The biotin and EDC solutions were then mixed 1:1 to achieve a final concentrartion of 25 mM solution. The probes were submerged in the biotin-EDC solution for 2 hours at room temperature and then incubated in the streptavidin-conjugated anti-CD63 primary antibody (Abcore, Cat. # AC12-0278-22) at a 1:1000 dilution at room temperature for 1 hour.

3.2 Exosome Purification

Exosomes were isolated from conditioned astrocytes media (ACM) using a total exosome isolation reagent (Thermo Fisher Scientific, Cat. # 4478359). This reagent forms a mesh around the exosomes that further pelleted using centrifugation from the rest of the cellular complement. This represents a control group of exosomes isolated using the traditional method that will serve as a basis for comparison with the ExoSense-isolated exosomes. Human astrocytes (ScienCell™ Cat. #1800) were cultured until 90% confluent according to the standard protocol using primary cell culture medium (TFS, Cat. #A1261301). The astrocytes released exosomes for 72 hours into the cell media before collection. The cell media was collected and centrifuged at 2000 G for 30 mins for pre-clearing of cell debris. The ACM was then transferred to a new tube and stored at -80 °C until further use. The ACM was allowed to thaw at room temperature and mixed with the isolation reagent at a ratio of 2:1 according to the protocol. The sample was vortexed and incubated at 4 °C for 24 hours. The exosomes were pelleted using centrifugation at 10,000 G for 1 hour at 4 °C. The supernatant was discarded, and the pellet resuspended in RIPA lysis buffer with protease inhibitors. A 7x protease stock solution was prepared by dissolving a cOmplete™ protease inhibitor cocktail tablet (Roche, Cat. # 04693124001)in 1.5 mL of D.I water. RIPA was prepared by mixing the protease inhibitor cocktail in a ratio of 1:7 according to the vendor recommendations.

The ExoSense protein lysate samples were prepared using 85 probes following the procedure described above. The exosomes capture pins were incubated for 16 hours at 4 °C in ACM. The exosomes were then lysed using the same RIPA solution as for the traditional sample, and the corresponding protein and RNA cargo were purified. A BCA

assay (Thermo Fisher Scientific, Cat. # 23225) was used to determine the protein concentration of the samples before use in the study.

3.3 Protein Concentration Assay

All protein samples including the total cell lysate protein, exosomes purified using polymer precipitate, and exosomes purified using fully functionalized probes were quantified using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Cat. # 23225). The BCA working reagent (WR) was prepared by mixing BCA Reagent A with BCA Reagent B at a 50:1 ratio respectively at room temperature. The protein standards (A-I) were prepared using RIPA lysis buffer (TFS, Cat. #89900) mixed with protease inhibitors (Roche, Cat. # 04693124001) as a diluent and stored at -20 °C until further use. The standard concentrations were prepared according to the protocol, using RIPA as the diluent.

The standard was prepared using the provided 2000 ng μL^{-1} bovine serum albumin (BSA) protein. The first two dilutions were prepared from the stock BSA solution and RIPA diluent to produce concentrations of 1500 ng μL^{-1} and 1000 ng μL^{-1} respectively. The remaining dilutions were prepared serially from the first two dilutions using RIPA as the diluent to create a standard curve with values of 750 ng μL^{-1}, 500 ng μL^{-1}, 250 ng μL^{-1}, 125 ng μL^{-1}, 25 ng μL^{-1}, and 0 ng μL^{-1} (blank, RIPA only). The standard curve was used to determine the concentration of the protein samples. The working volume of the samples was reduced to 2 uL from the original protocol to adjust for the requirements of the NanoDrop™ reader. The WR was mixed at a 1:1 ratio for each standard and sample measured (3 µL with 3 µL), vortexed, heated at 65 °C for 5

minutes using a heat block, and the absorbance was measured at 562 nm using a NanoDrop™ 2000 (Thermo Fisher) spectrophotometer.

3.4 Fluorescent Imaging Analysis

Fully functionalized microprobes were prepared using the protocol described above. Once functionalized, the probes were incubated in cell media for 16 hours at 4 °C with agitation on an orbital shaker. For the fluorescent imaging analysis 6 experimental groups were prepared: CD63, CD63 + 0.1% BSA, Biotin, Biotin + 0.1% BSA, Negative Control and Negative Control + 0.1% BSA. The CD63 group was functionalized with anti-CD63 antibodies for exosome capture. The efficiency of BSA as a blocking reagent was investigated after 20 minutes of incubation in a 0.1% BSA solution following the antibody immobilization.

Control experiments in this analysis will be performed by using biotin-only coated probes for comparison with the anti-CD63 fully functionalized microprobes. The biotin-only probes are expected to have none or a very low level of fluorescence while the CD63 functionalized probes are expected to produce a uniform signal. The Biotin only group serves as a control that does not include anti-CD63 antibodies. These control experiments were included to determine the rate of non-specific exosome adherence to the biotin-covered surface of the probes in the presence and absence of 0.1% BSA blocking reagent. Before administering the blocking reagent, 0.1% BSA was also used as a wash buffer by dipping the probes into the buffer and allowing them to air dry before continuing to the next step. The negative control groups were included to test the degree of nonspecific fluorescence signal. For these control experiments, fully functionalized

probes were incubated with primary and secondary detection antibodies while the cell media incubation step was omitted.

Following the preparation of all 6 groups, the probes were incubated in a mixture of CD9 and CD81 primary antibodies (R&D Systems, Cat. #MAB25292, Cat. #MAB46151) at a concentration of 0.05 µg μL^{-1}, per vendor recommendation, using 1x phosphate-buffered saline (PBS) as a buffered diluent for 1 hour at room temperature. The groups were then dipped in a 0.1% BSA solution for a quick wash, allowed to dry, and incubated in NL557-conjugate fluorescent secondary antibody (R&D Systems, Cat. #NL007) at a concentration of 0.005 µg μL^{-1}, per the vendor recommendation, for 1 hour at room temperature in the dark. Another quick wash was performed following the secondary antibody incubation step, allowing the probes to air dry before imaging. All probes were imaged using the 557 red filters at a 16% exposure in the EVOS FL instrument (Thermo Fisher Scientific) with the RFP LED cube. The images were analyzed using Image J software by manually highlighting the area of the probe exhibiting fluorescence and measuring, setting the software to include area, integrated intensity, and mean gray value in the measurement. Following the measurement of the fluorescence, an area on the probe that did not exhibit fluorescence was highlighted, at the same size as the fluorescence area, to establish the "blank" fluorescence, keeping the measurement settings the same in the software. The fluorescence and blank measurements were moved into excel to calculate the corrected total cell fluorescence (CTCF) using the formula: Integrated Density – (Area of selected cell * Mean fluorescence of blank readings). Four images were analyzed for each probe and five probes were analyzed per treatment group. 1-6 fluorescence measurements were taken

per image, depending on the coverage of fluorescence on the probe (Appendix A). 3 blank measurements were taken for every fluorescence measurement. The mean value was calculated for the fluorescence measurements, and the blank measurements before using the CTCF calculation. Results from the fluorescent probe analysis will be utilized to assess the specificity of the solid phase microprobe purification technique for exosome isolation.

3.5 SDS-PAGE

The total protein, exosome polymer reagent, and exosome probe proteins were allowed to thaw from -20 °C storage to room temperature and were mixed at a 1:1 ratio with Lameli buffer. The concentration of each protein sample was 2 µg μL^{-1}. All protein samples were denatured at 90 °C for 8 minutes, allowed to cool back down to room temperature to avoid melting the gel, and then loaded into a stain-free polyacrylamide gel (BioRad, Cat. #4561093) for electrophoresis (4-20%) at a concentration of 20 µg per lane. The SDS buffer was prepared by mixing 10x Tris/Glycine/SDS Buffer (BioRad, Cat.#1610732) with D.I water at a ratio of 1:10. The gel was loaded into the electrophoresis chamber with the protein samples alongside a BioRad Kaleidoscope Protein Standard (Cat. #1610375) on either side of the protein lanes. Electrophoresis was performed at 90 V for 55 minutes until the samples and the standard reached the end of the gel. The gel was imaged using a BioRad ChemiDoc Imaging System, after 45 seconds of exposure to UV light.

3.6 Western Blot Analysis

The expression levels of two different classes of proteins: CD63 (exosome-specific) and Rab5 (endosome-specific) will be measured via Western Blot analysis. The

presence of these proteins will be used to exclude the possible presence of membrane vesicles derived from membrane shedding and disruption (Logazzi et al., 2009). The presence of canonical exosome proteins (CD63) and endosome-specific Rab5 will demonstrate a pure exosome preparation (Lobb et al., 2015) that can be used for comparative proteomic analysis.

The protein samples (total cell lysate and exosomes purified using polymer precipitation) were thawed from -20 °C storage to room temperature and measured in concentration using the BCA assay previously described. The total cell lysate sample was prepared by using 30 uL of RIPA buffer mixed 1:7 with protease inhibitors directly on the surface of cultured astrocytes. The astrocytes were seeded in a T-75 flask and had reached confluency when the proteins were extracted ($8.4 * 10^6$ cells).

SDS-PAGE was performed before blotting the proteins onto the membrane for Western blot analysis. SDS-Buffer was prepared by mixing BioRad 10x Tris/Glycine/SDS Buffer (Cat. #1610732) with D.I water at a ratio of 1:10 according to the vendor recommendations. All protein samples were loaded into a stain-free polyacrylamide gel (4-20%, BioRad, Cat. #4561093) at a concentration of 20 µg per well as follows from left to right: Standard, Exosome, Control, Standard, Exosome, Control, Standard, Exosome, Control, Empty. Standard refers to BioRad Kaleidoscope Protein Standard (Cat. #1610375) and was loaded using 5 µL per well. Electrophoresis was run on the gel at 80 V for 20 minutes. The gel was then equilibrated in transfer buffer for 15 minutes, and the blotting sandwich was then assembled using a standard cassette as follows: fiber pad, blotting paper, gel, nitrocellulose membrane (BioRad, Cat. #166-2807), blotting paper, and fiber pad. The gel was set against the negative electrode, while

the membrane faced the positive electrode, and a roller was used to remove bubbles in the blotting sandwich. Transfer buffer was prepared by mixing 200 mL of 10x TrisGlycine buffer (BioRad, Cat. #1610734) with 400 mL of methanol (VWR, Cat. #BDH1135-1LP) and 1400 mL of D.I water. Electrophoresis in the transfer buffer was run at 60 V for 1.5 hours with an ice pack in the module to prevent overheating of the gel.

Following the transfer step, Pierce™ western blot signal enhancer (Thermo Fisher, Cat. #21050) was used according to the vendor recommended protocol. The membrane was rinsed with D.I. water briefly and incubated in 10 mL of Reagent A at room temperature for 2 minutes with agitation. The membrane was briefly washed five times in D.I. water and incubated in Reagent B for 2 minutes at room temperature with agitation. The membrane was washed five times in D.I. water and the blocking step was performed.

For the blocking step, the membrane was blocked using a nonfat dry milk blotting buffer (BioRad, Cat. #1706404) prepared by mixing 3 g of dry milk with 6 mL of 10x PBS (BioRad, Cat. #1610780) and 54 mL of D.I water. The membrane was blocked for 1 hour at room temperature with agitation using 20 mL of the blocking buffer. The membrane was cut horizontally with a standard, exosome sample (polymer precipitation), and control (total cell lysate) sample on each side of the membrane. CD63 and RAB5 primary antibodies (Proteintech®, Cat. #25682-I-AP, Cat. #11947-I-AP) were each prepared at a 1:500 dilution in the blocking buffer, adding Tween 20 (BioRad, Cat. #1610781) at 0.1% of the solution. The membranes were incubated in 6 mL of the primary antibody overnight (14 hours) at 4 °C with agitation. Following the primary incubation, each membrane was briefly washed in wash buffer and then washed four

times for 5 minutes each with agitation in 8 mL of wash buffer. The wash buffer was prepared by mixing 449.5 mL of D.I Water with 50 mL of 10x PBS and 500 uL of 10% Tween 20. The secondary antibody (Invitrogen, Cat. #31460) was prepared at a 1:10,000 dilution in the blocking buffer with Tween 20 added at a final concentration of 0.2%. The membranes were incubated in 6 mL of the secondary antibody for 1.5 hours at room temperature with agitation. Following the secondary incubation, the wash steps were repeated, and the membranes incubated in SuperSignal™ West Femto chemiluminescent substrate (Thermo Fisher, Cat. #34094) for 2 minutes, prepared according to the vendor recommended protocol. Once incubated, the membrane was imaged using BioRad ChemiDoc Imaging System after 60 seconds of UV exposure and the image was overlayed with a colorimetric image of the standard.

CHAPTER 4

RESULTS

The ExoSense microprobe capture technology is based on an antigen-specific interaction with CD63, a tetraspanin surface protein that is unique to exosomes. Because CD63 is a confirmed surface marker for exosomes (Mathieu et al., 2019), it is hypothesized that the probes are capable of selectively isolating exosomes from biological samples. High selectivity of exosome capture has already been observed with the use of antigen-specific Dynabeads™ (Doyle and Wang 2019), however, the high-salt buffer required for removing the vesicles from the beads destroys the recovered sample. Our technology reduces the procedure for exosome capture down to a single step, offering the ability to analyze exosome contents directly from the probes without introducing salt contaminants or other potentially destructive procedural steps. The main objective of these experiments was to compare the exosomal contents of a probe-captured sample with a traditional polymer precipitation method of exosome isolation using SDS-PAGE and to characterize the exosomes on the surface of the probes using fluorescent imaging analysis to validate our hypothesis concerning exosome-directed CD63 enrichment on the surface of the ExoSense microprobes.

4.1 SDS-PAGE

The lanes of the gel depict the following groups from left to right: Std., exosome probe sample, exosome polymer reagent sample, and Std, with Std. indicating the Biorad Kalidescope™ protein standard. (Figure 4.1). A 45 second exposure time was used to optimally show the protein bands for each sample. A gel image with a 10-second exposure was merged with the 45 second image to more accurately identify the molecular weights of each band. Protein bands in the 20-25 kDa range were detected in all three experimental groups. Bands with a molecular weight of around 65 kDa were observed in the probe samples only. A protein band around the 72 kD marker was not visible within the probe protein sample and is faintly visible within the polymer reagent and total protein groups. Overall, the SDS-PAGE results depict fewer bands in the probe sample when compared to the polymer reagent and total protein groups.

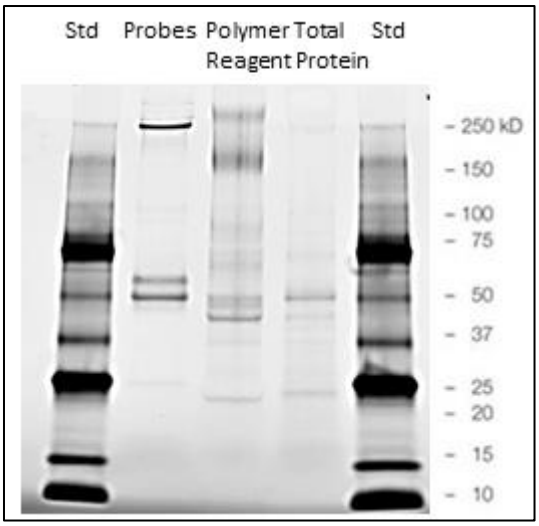


Figure 4.1: Comparison of exosome isolation methods via SDS-PAGE analysis. BioRad Kalediscope™ protein standard was loaded to the left and right of the protein samples as a clearly defined molecular weight scale, and the protein sizes are indicated at the right of the figure (kD). From left to right following the standard, the protein samples were loaded as: exosomes captured using probes, exosomes purified using the polymer precipitation reagent, and the total cell lysate. All protein samples were prepared at a 1:1 ratio with Lameli buffer and loaded at a concentration of 20 µg per well.

4.2 Fluorescent Imaging

The fluorescent analysis resulted in 6 clearly defined groups (Figure 4.2). The CD63 group expressed the most fluorescence of all of the groups and shows a significant difference when compared to the Biotin and Negative Control groups. Fluorescence was detected in the Biotin groups, indicating a certain level of non-specific binding of exosomes to the probes. Finally, the Negative Control groups indicate a negligible amount of fluorescence detected, likely contributed by the non-specific binding of the fluorescent antibodies to the probes. Each group can be accurately assessed to predict and validate the specificity of exosome capture by the probes.

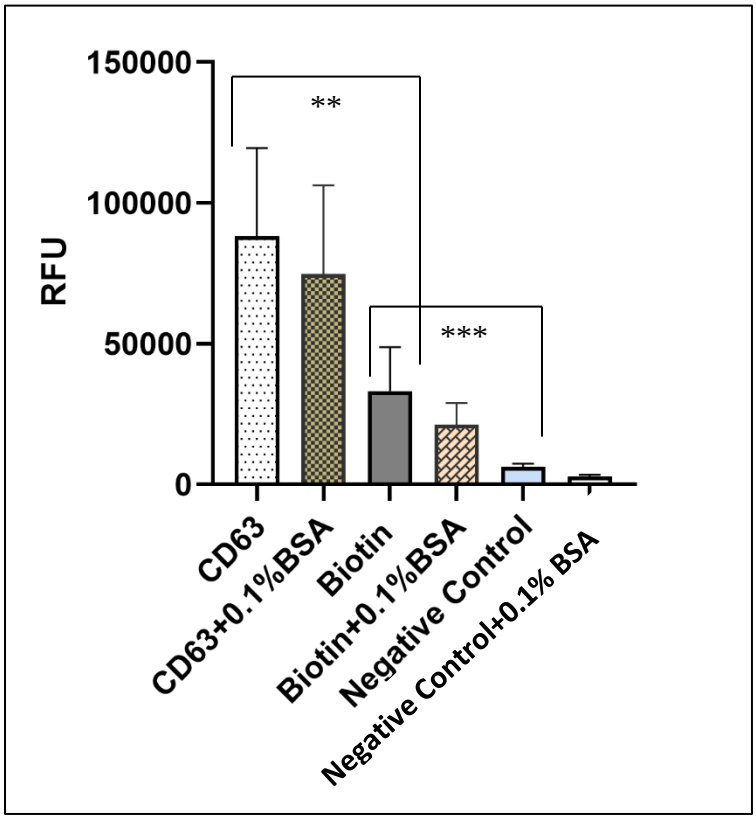


Figure 4.2: Corrected Total Cell Fluorescence (CTCF) of functionalized microprobes for specificity analysis. 6 groups of probes (CD63, CD63 + 0.1% BSA, Biotin, Biotin + 0.1% BSA, Negative Control, Negative Control + 0.1% BSA) were immobilized with anti-CD9 primary antibodies and secondary 557 nm fluorescent antibodies and imaged using an EVOS fluorescent imaging machine with RFP LED cube, 16% exposure saturation. Images were analyzed using Image J and calculated in excel with the CTCF formula. P-values resulting from Wilcoxon Rank Sum (Mann Whitney-U test for distributions of unequal variance).

4.3 Western Blot

Protein samples from polmer precipitated exosomes, BioRad protein standard, and total cell protein were loaded into a polyacrylamide gel and transfered to a nitrocellulose membrane. The membrane was then cut in half vertically with one half prepared with CD63 and the other half with RAB5 primary antibodies according to the previously described protocol. Both membranes were imaged using 60 seconds of chemiluminescent exposure and merged with a colorimetric image to visualize the bands of the standard. The groups appear on the membranes (placed side by side for imaging) as follows on the CD63 side: exosome sample, total cell control protein, and on the RAB5 side: protein standard, exosome sample, total cell control protein. No clear bands are visible in the control proteins (Figure 4.3). One faint band is visible in each membrane of the polymer precipitated exosome samples, at 23 kDa and 24 kDa respectively. These are the expected molecular weights described by the primary antibodies each membrane was incubated in, suggesting the presence of CD63 and RAB5 antigen enrichment in the samples. A thick band is present at the 150 kDa mark and can be indicative of non-specific binding by the primary antibodies.

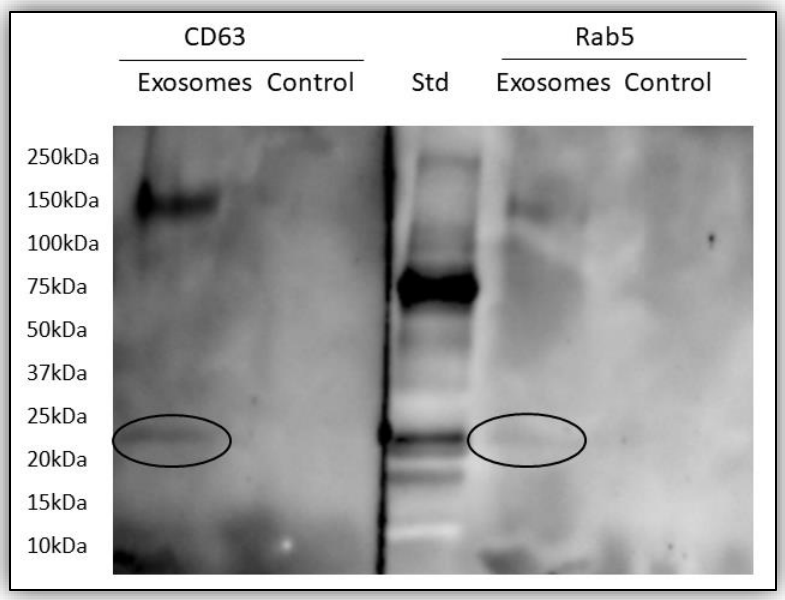


Figure 4.3: CD63 and RAB5 Western blot membrane. Nitrocellulose membrane loaded with 20 µg per lane and cut in half lengthwise following blocking step. Primary incubation in CD63 and RAB5, 1:500 dilution in blocking buffer. Secondary incubation 1:10,000. Faint bands appear on each membrane at the 20-25 kDa range, indicating both CD63 and RAB5 positive antigens in the exosome (polymer precipitation) sample.

CHAPTER 5

DISCUSSION

5.1 SDS-PAGE protein profile

SDS-PAGE separation was performed to assess the protein bands profile of exosomes purified via traditional polymer precipitation, exosomes enriched with the ExoSense microprobe, and total protein from cell lysate. The gel image depicts fewer bands in the probe-derived sample when compared to the polymer-precipitated and total protein groups (Figure 4.1). The total cell lysate is expected to be composed of a larger variety of proteins when compared to the exosomal samples. The presence of more and thicker bands in the polymer-precipitated exosomal sample compared to the ExoSense probe is probably due to co-precipitation of non-exosomal proteins, which is a commonly reported issue. The probe-derived exosomal sample displayed three distinct protein bands of approximately 50 kDa, 60 kDa, and 250 kDa. The protein band in the 20-25 kDa range in the SDS-PAGE gel image is more pronounced in the total cell lysate sample while being faint in the polymer-precipitated sample, and barely visible in the probe-isolated protein lysate. Because all protein samples were measured and loaded at the same concentration (20 µg per lane), the thicker bands reflect the cumulative amount of similar-sized proteins, which are more heterogeneous in the total cell lysate and more uniform in the probe-isolates. These bands could be indicative of CD63 or RAB5 proteins

associated with exosomes as they fall into this molecular weight range, however, the identity of these bands cannot be confirmed with SDS-PAGE alone. The fluorescent imaging analysis can be used to confirm the enrichment of CD63 proteins from the probes.

The probe sample gives the appearance of four distinct protein bands as well as the faded appearance of two additional bands. The highly concentrated protein band at the 67 kDa mark is most likely bovine serum albumin (BSA), collected in the sample residually from the 0.1% BSA blocking step of the probe preparation. Although the identity of the 67 kDa protein band cannot be confirmed from the SDS-PAGE analysis, it is understandable that BSA from the probes would be lysed by the RIPA buffer alongside the exosome proteins considering that the BSA-probe bond is relatively weak. The collection of residual BSA is a relevant factor to consider in the optimization process of the microprobe protocol for application purposes.

Because all protein samples were measured and loaded at the same concentration (20 µg per lane), the strength of the bands likely reflects the specificity of proteins collected in each sample. For instance, previous research indicates that all samples are expected to have a protein marker in this range (Kowal et al., 2016), but the total protein sample should contain a larger variety of proteins than the polymer precipitation and probe samples because exosomes contain fewer proteins than the cell as a whole (Valadi et al., 2007). The presence of more, stronger bands in the polymer reagent sample compared to the probe sample is likely indicative of excess proteins or cell lysate precipitated in the polymer reagent sample along with the exosomes while the fainter, fewer bands in the probe sample likely reflects higher specificity of proteins collected by

the probes. This is also supported by the faintly visible band that is present in the polymer reagent sample around 72 kD, which could indicate the presence of Calnexin, a protein not present in exosome samples (Matheiu et al., 2019) that likely appeared from excess proteins collected in the precipitate. This band is not visible in the probe sample, further supporting the exosome-specificity proteins collected by the probes.

5.2 Specifically defined fluorescence groups

The fluorescent imaging analysis (Figure 4.2) shows distinct differences between all 6 groups prepared. The CD63 group expressed the most fluorescence because it was functionalized with the anti-CD63 antibody, enabling the enrichment of exosomes by the probes and therefore a larger surface area for the CD81 and CD9 fluorescent antibodies to bind to. The blocking reagent reduced the fluorescent signal for each group, indicating that the 0.1% BSA solution reduced the presence of non-specifically adhered fluorescent antibodies on the probes. The negative control groups can be used to subtract the non-specific signal obtained from the fluorescent antibodies, and the Biotin groups can be used to subtract the non-specific signal obtained by the probes, yielding a more accurate estimate for the capture ability of the probes overall. After removing the non-specific signals detected by all other groups, the CD63 + 0.1% BSA group still exhibits a significant fluorescent signal, supporting our hypothesis that the ExoSense probes provide specific and efficient exosome capture.

5.3 Impact of exosomal sample method

The traditional Dynabeads™ immunosolation method for exosome purification includes an enrichment step to increase the concentration and purity of collected CD63+ exosomes. However, enriching an exosome population from a biological sample typically

involves precipitating the exosomes using a commercially available enrichment reagent that increased the cost and duration of the procedure. Our method of using microprobes allows for the direct capture of exosomes from cell media, potentially providing a pure exosome population but at a significantly lower concentration than what the enrichment step yields. Direct exosome isolation from cell media also presents an increased exposure to excess particulates, potentially interfering with the concentration of pure exosome populations obtained, as compared to other studies (Dash et al., 2020). The SDS-PAGE analysis suggests that the exosome population isolated using the microprobes is higher in purity than the exosomes isolated using the polymer precipitation reagent, as there are more bands present in this group (Figure 4.1). Excess particulates' interference can potentially be accounted for with the use of the blocking step on the microprobes. However, the concentration of populations isolated will require optimization to account for the number of microprobes necessary for sufficient exosome extraction.

5.4 The efficiency of BSA blocking

The blocking step (0.1% BSA) was included to evaluate the specificity of each experimental group. This additional step was included to reduce the possibility of nonspecific binding by the fluorescent antibodies and captured microvesicles on the probes. Although the T-test indicates that these 0.1% BSA groups are not statistically significant, there is still a noteworthy effect exhibited by the treatment groups. For each treatment group where 0.1% BSA was included, the fluorescent signal was considerably reduced which points to the presence of nonspecific binding on the probes. A lack of statistical significance may indicate that the level of nonspecific binding is considerable

and should be further evaluated during the optimization stage of the microprobes for standardized commercial use.

5.5 Western blot analysis

The Western blot results are indicative of a protocol that may not be fully optimized for exosome protein samples. Several factors should be considered in the optimization process of this protocol that includes exosomal protein and antibodies concentrations as well as blocking reagents optimization. The exosome protein content is very low and more homogeneous when compared with a traditional sample. The amount of protein loaded per lane likely needs to be higher for exosome samples to account for the lower concentration in the final bands. The higher background noise may be related to interactions of the specific primary or secondary antibodies with the blocking buffer, therefore, better signal-to-noise images can be obtained after further adjustments of the conditions. Bands in the 20-25 kDa range (Figure 4.3) are indicative of CD63 and RAB5 antigens present in the exosome samples and could be used to confirm the identity of specific proteins from exosome samples upon optimization of the protocol showing a clearer result.

Western blot results were not obtained from the ExoSense probe collected exosome samples. The protein samples were lysed directly from the probes, most likely leaving the surface membrane tetraspanin proteins on the probes' surface. Only residual tetraspanins left in the protein sample are not enough to confirm the presence of this marker from the probe sample via Western blot. It has also not yet been confirmed that CD63 proteins can be present within exosome samples, therefore, the best method for confirming the enrichment of CD63 by the ExoSense probes is through fluorescently

labeled antibody imaging and analysis on the surface of the probes, as indicated in this study (Figure 4.2).

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 Conclusions and future directions

The reduced bands in the probe sample of the SDS-PAGE gel when compared to the polymer reagent and total protein samples demonstrate the reduced capture of proteins and therefore support the use of the probes as a more specific method of exosome capture than the traditional precipitate method. CD63 is confirmed to be an exosome-specific protein (Matheiu et al., 2019). Therefore, the fluorescent imaging analysis yielding a significant signal after the removal of non-specific signals indicates an exosome-specific capture ability of the probes. Together, the SDS-PAGE and fluorescent imaging analysis results validate the microprobes as a highly specific, efficient method of exosome capture compared to the traditional capture method.

However, a more specific method means the amount of protein collected has to be much higher before results can be depicted using traditional proteomic studies. Future work should focus on the application of the microprobes using more complex biological samples, such as mouse serum or blood, to evaluate the specificity. The results of this study yield a significant contribution to the field because they support the use of microprobes as a highly specific, noninvasive method of exosome capture from biological samples and carry the potential to accelerate the study of exosomes in molecular biology research as a whole.

APPENDIX A : FLOURESCENT PROBE IMAGES

Exemplary images from each treatment group of the fluorescent probe analysis (Figure 4.2) are shown.

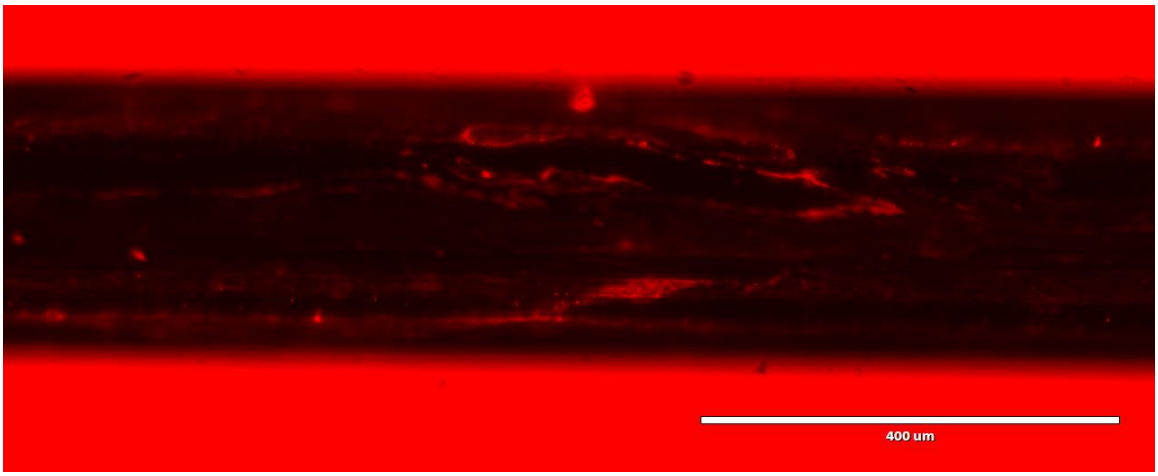


Figure A1: Fluorescent probe image from CD63 treatment group. Fully functionalized probe, 400 µm scale.

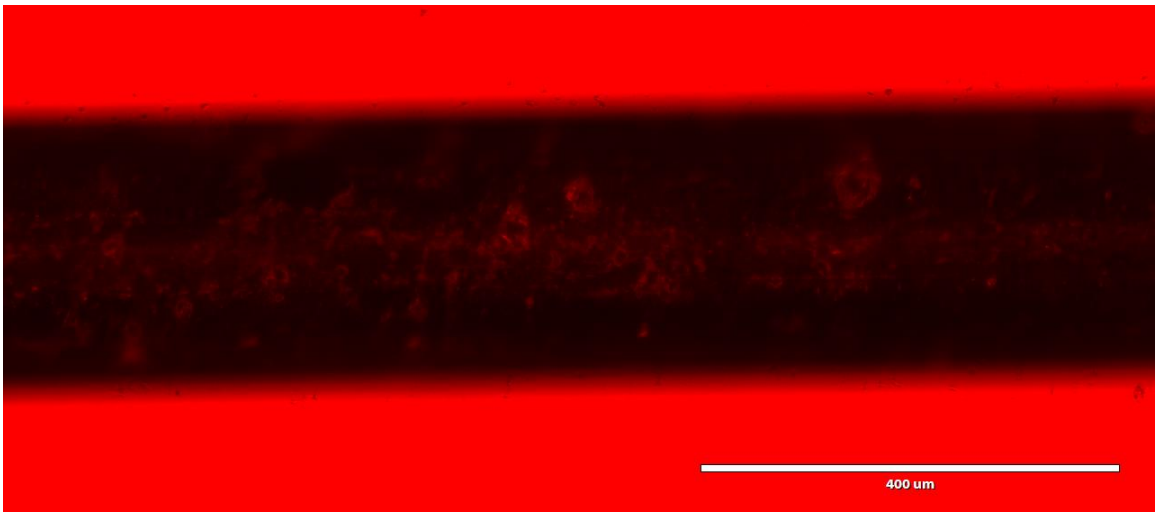


Figure A2: Flourescent probe image from CD63 + 0.1% BSA group. Fully functionalized probe plus blocking step, 400 µm scale.

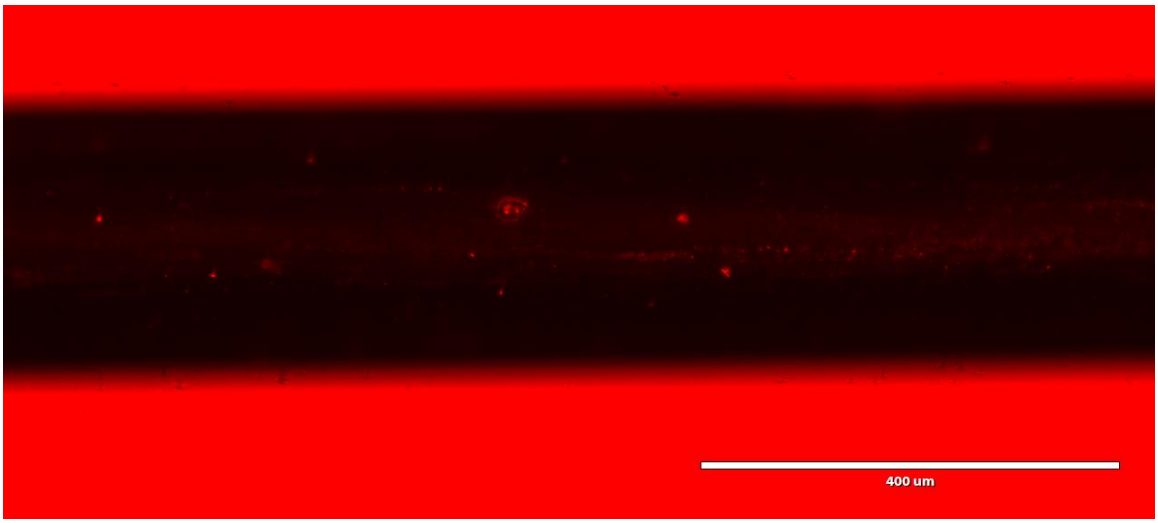


Figure A3: Fluorescent probe image from Biotin group. Biotin-only functionalization, 400 µm scale.

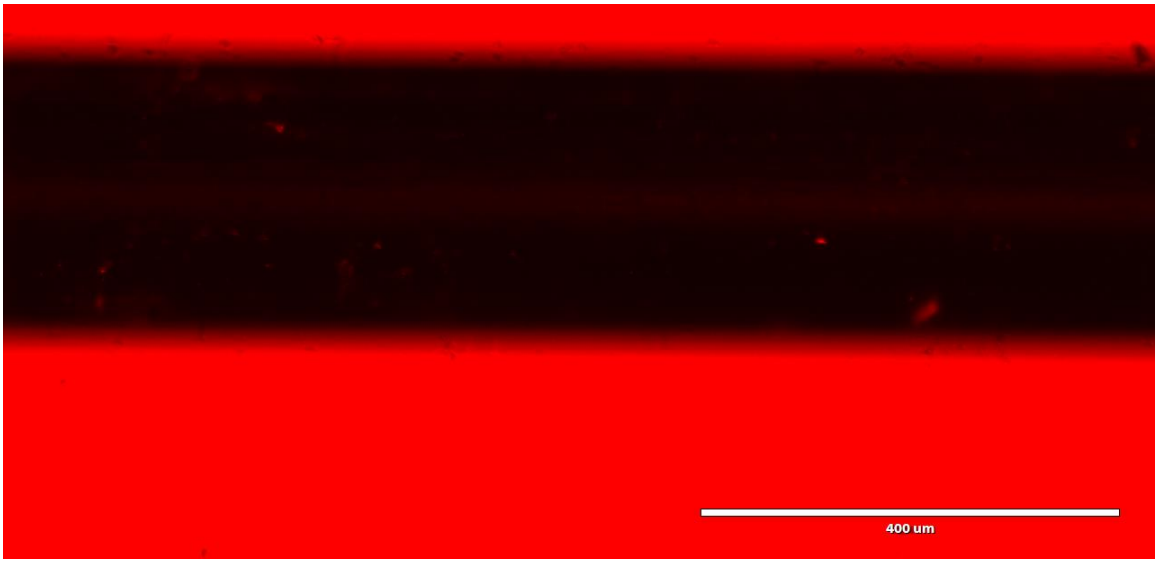


Figure A4: Fluorescent probe image from Biotin + 0.1% BSA group. Biotin-only functionalization plus blocking step, 400 µm scale.

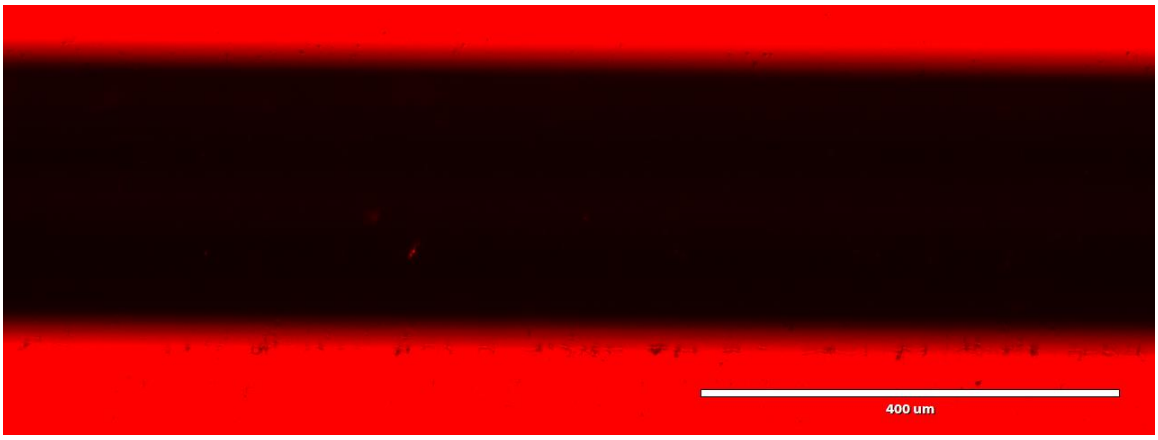


Figure A5: Fluorescent probe image from Negative Control group. Biotin-only functionalization plus the omission of cell media incubation, 400 µm scale.

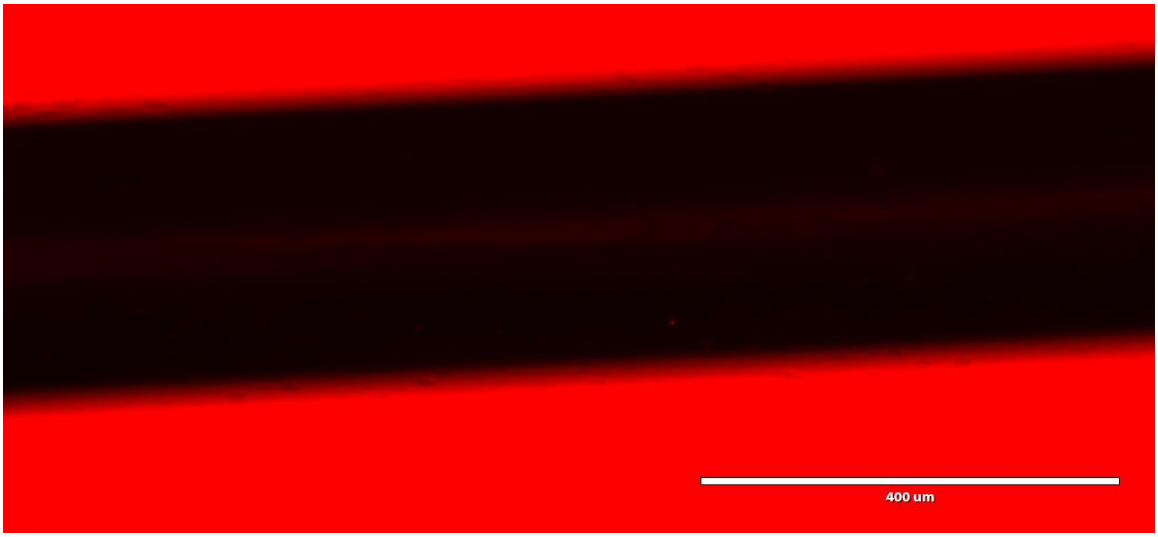
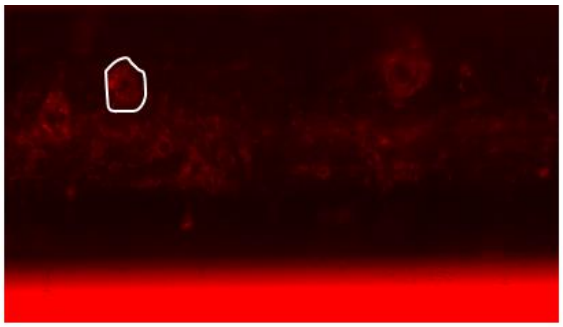


Figure A6: Flourescent probe image from Negative Control + 0.1% BSA group. Biotin-only functionalization plus omission of cell media incubation plus blocking step, 400 µm scale.

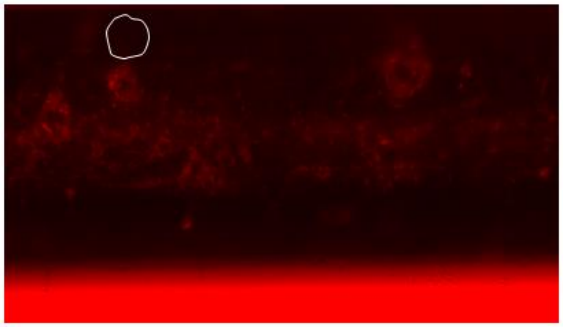
APPENDIX B : FLUORESCENT PROBE IMAGE ANALYSIS PROTOCOL

A flowchart to describe the Image J analysis protocol used to determine CTCF (Figure 5) is included below.

Highlight Area of Fluorescence



Highlight Area of "Blank" Fluorescence



Copy Image J Measurements into Excel

AB FP 1		Selcted Area	Mean Value	Integrated Density	Flourescent or Blank?
	1	986	21.273	20975	F
	2	68	7.485	509	B
	3	100	7.14	714	B
	4	62	6.371	395	B

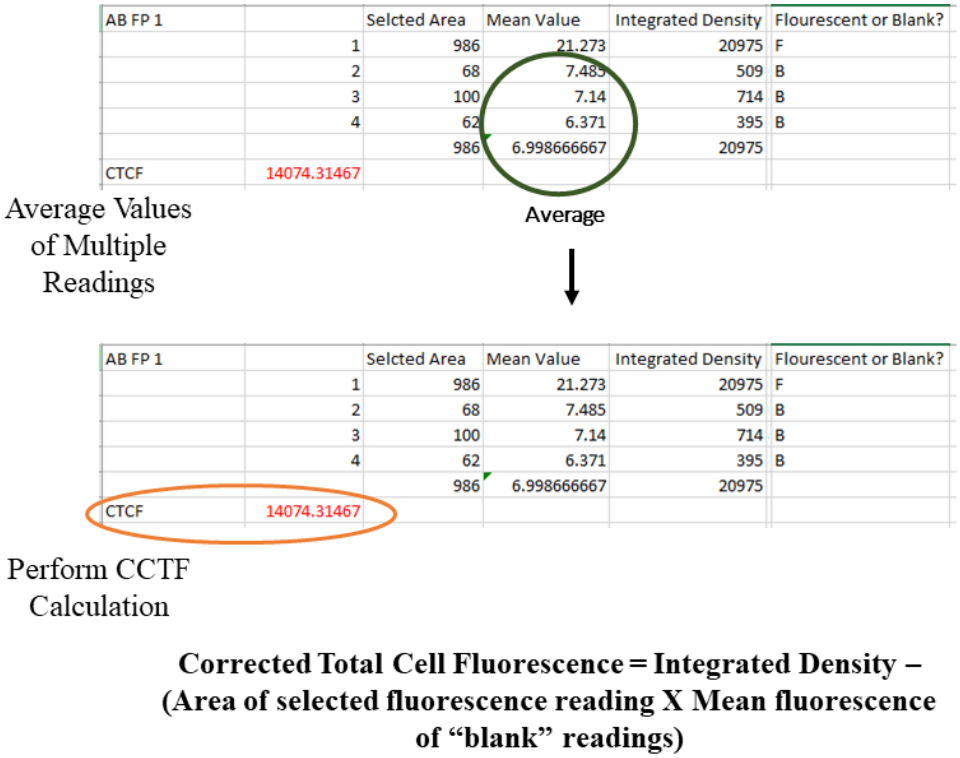


Figure B1: Flowchart for visual Image J analysis protocol. Flourescent and blank measurements were taken within the Image J software and copied into Excel for calculations. Multiple values were averaged together and the CTCF calculation was performed.

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