Development of Dual Imaging Single Vesicle Technology for Exosome Characterization and Early Cancer Detection

Kristopher Amrhein

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DEVELOPMENT OF DUAL IMAGING SINGLE VESICLE TECHNOLOGY FOR
EXOSOME CHARACTERIZATIONS AND EARLY CANCER DETECTION

By

Kristopher D. Amrhein

A Dissertation submitted in
Partial Fulfillment of the
Requirements for the
Degree of Doctor of Philosophy

Major: Chemistry

The University of Memphis
May 2023
DEDICATION

This dissertation is dedicated to my family and friends who have been with me throughout all of the ups and downs that my life and academic career has offered. To my parents, George and Kathy Amrhein, without them, I would have never made it as far as I have. Their love and support has helped guide me through this process, and I will always do my best to keep making them proud. To my best friend of almost 30 years, Matthew Moorhouse, without your friendship and inspiration, I would have never been able to persevere through many of the obstacles life has put in my path, and one day I hope to be as successful as you have been with life’s gift.

Thank you to everyone for the love and support.
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Secondly, I would like to thank my committee members, specifically Dr. Thang Ba Hoang and Dr. Yongmei Wang, for their continued support throughout my graduate school experience. With them, I was successful at seeing my potential in areas with their unique expertise, which has given me great insight into my research and allowed me to have a multidisciplinary perspective of my work. Finally, I am also extremely grateful and would like to thank Dr. Michael Brown and Dr. Daniel Nascimento for allowing me to take time from their busy schedules to be on my committee to help guide me through the final stages of my graduate career.

Thirdly, I would like to show my gratitude and appreciation to my former lab members: Dr. Ryan Timothy O'Connor, Dr. Elyahb Allie Kwizera, Dr. Vojtech Vinduska, Dr. Raymond Edward Wilson, and Zechariah J. Avello who were not only mentors, guiding me in uncountable matters related to our research, but also lifelong friends. In addition, I would like to thank Alberto Luis Rodriguez-Nievez, Mitchell Lee Taylor, and Anthony Gregory Giacalone for their dependable support and friendship throughout this process. Furthermore, I would also like to thank
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PREFACE

The writing of this dissertation is based on one review article and one journal article:

Chapter 1 is designed to be an overview of cancer and exosomes as well as their use in biomedical detection. First, a background for cancer and the significance of early detection in breast cancer is given. Then, some essential physical and quantitative qualities of exosomes and their applications for characterization and detection are discussed. Further, several examples of their use in biomarker detection are presented for consideration.

Chapter 2 is a review of current exosome detection and analysis technologies with gold nanoparticles, which is partially based on a review article published in the journal of Nanomaterials: “Taylor, M.T., Giacalone, G.G., Amrhein, K.D., Wilson Jr, R.E., Wang, Y., and Huang, X. Nanomaterials for Molecular Detection and Analysis of Extracellular Vesicles. Nanomaterials 2023, 3(13), 524.” In this chapter, all figures, schematics, and references have been reformatted and renumbered to fit into one document. The references and style used within this chapter reflect the standards of the Journal of Nanomaterials.

Chapters 3 to 5 are based upon a journal article published in the journal of ACS Applied Materials and Interfaces: “Amrhein, K., Taylor M.L., Wilson, R., Gallops, C.E., Annamer, A., Vinduska, V., Kwizera, E.A., Zhang, H., Wang, Y., Hoang, T.B., and Huang, X. Dual Imaging Single Vesicle Surface Protein Profiling and Early Cancer Detection. ACS Applied Materials and Interfaces 2023, 15(2), 2679-2692” In this chapter, all figures, schematics, and references have been reformatted and renumbered to fit into one document. The references and style used within this chapter reflect the ACS Applied Materials and Interfaces standards.

Chapter 6 is a summary of all the contents covered in this dissertation.
ABSTRACT


Single vesicle molecular profiling has the potential to transform cancer detection and monitoring by precisely probing cancer-associated exosomes in the presence of normal exosomes in body fluids. However, it is challenging due to the small exosome size, low abundance of antigens on individual exosomes, and complex biological matrix. A facile dual imaging single vesicle technology (DISVT) has been developed and described for surface protein profiling of individual exosomes and quantification of target-specific exosome subtypes in biofluids by integrating multiple components, including direct molecular exosome capture from diluted biofluids, dual exosome-protein light scattering/fluorescence imaging, and fast Python-programing based image analysis. This technology uses strong surface plasmon light scattering properties of gold nanoparticles (AuNPs) to detect targeted surface protein markers with darkfield imaging on exosomes at the single particle level. Meanwhile, fluorescent imaging uses a fluorescent near-infrared dye to localize captured exosomes. A customized microscope achieved dual light scattering/fluorescence imaging. By optimizing various parameters, including concentration, binding time, and binding temperature of the AuNPs and dye, this DISVT can detect targeted surface protein markers of interest at the single vesicle level.

Using DISVT, we characterized several protein markers on exosomes derived from several model breast cancer (BC) cells, validating them with standard ELISA. We demonstrated the potential of DISVT for early cancer detection using Human epidermal growth factor receptor 2 (HER2)-positive BC as the disease model by profiling HER2-positive exosomes in plasma from HER2-positive BC patients at multiple stages, comparing them with healthy donors and HER2-
negative BC patients. We demonstrated that the DISVT, but not ELISA, detected BC at early stages. The DISVT differentiated HER2-positive- from HER2-negative- BC. The amount of tumor-associated exosomes showed to be tripled in locally advanced- compared to early-stage- patients. Our studies suggest that single exosome surface protein profiling with DISVT can provide a novel and highly sensitive method for early cancer detection and quantitative monitoring. Due to the advantages in simplicity, sample consumption, speed, and cost, this DISVT has the potential to accelerate the translation of exosomes into clinical use, leading to a new generation of liquid biopsy for detecting and monitoring cancer and other diseases.
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LIST OF SYMBOLS AND ABBREVIATIONS

~ Approximately

= Equal to

> Greater than

\leq Less than or equal to

\bar{\xi}_p Average normalized pixel intensity of the DF images

\lambda Wavelength

° Degrees

°C Degrees Celsius

\mu g/mL Microgram per milliliter

\mu L Microliter

\mu m Micrometer

\mu NMR Micro-Nuclear Magnetic Resonance

2D Two dimensional

3D Three dimensional

Ab Antibody

Ag Silver

AgNP Silver nanoparticle

ANOVA Analysis of Variance

Au Gold

AUC Area under the curve

AuNP Gold nanoparticle

AuNR Gold nanorod
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<tr>
<td>BC</td>
<td>Breast Cancer</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>Cy5</td>
<td>Cyanine-5</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast cancer gene</td>
</tr>
<tr>
<td>CCV</td>
<td>Clathrin-coated vesicles</td>
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<tr>
<td>CCD</td>
<td>Charge-Coupled Device</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CD81-PEG-SH</td>
<td>CD81-polyethylene glycol-thiol</td>
</tr>
<tr>
<td>cfDNA</td>
<td>Cell-free deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein Succinimidyl Ester</td>
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<tr>
<td>CLS</td>
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<td>cm</td>
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<td>Dynamic light scattering</td>
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<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate buffer solution</td>
</tr>
</tbody>
</table>
DPBST 0.01%  Dulbecco’s phosphate buffer solution with 0.01% (w/v) Tween 20
DPBST 0.05%  Dulbecco’s phosphate buffer solution with 0.05% (w/v) Tween 20
DMEM      Dulbecco’s modified Eagle’s medium
dsRNA     Double-stranded deoxyribonucleic acid
ELISA     Enzyme-linked immunosorbent assay
EpCAM     Epithelial cell adhesion molecule
EphA2     Ephrin type-A receptor 2
EGFR      Epidermal growth factor receptor
ER        Estrogen receptor
EV        Extracellular vesicle
exo-Anx II Exosomal protein Annexin II
FBS       Fetal bovine serum
FC        Flow Cytometry
FN        Fibronectin
$F_p$     Fraction of marker-positive
G         Grade
g/mL      Gram per milliliter
h         Hour
$\text{H}_2\text{O}_2$ Hydrogen peroxide
$\text{H}_2\text{SO}_4$ Sulfuric acid
HAuCl₄    Gold (III) chloride trihydrate
HCC       Hepatic cell carcinoma
HD        Hydrodynamic
<table>
<thead>
<tr>
<th>Abbr</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>He</td>
<td>Helium</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HER</td>
<td>Human epidermal growth factor</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>ILV</td>
<td>Intraluminal vesicle</td>
</tr>
<tr>
<td>iNPs</td>
<td>Intravesicular nanoplasmic system</td>
</tr>
<tr>
<td>kV</td>
<td>Kilovolt</td>
</tr>
<tr>
<td>LFA</td>
<td>Lateral flow assay</td>
</tr>
<tr>
<td>LFIA</td>
<td>Lateral flow immunoassay</td>
</tr>
<tr>
<td>lncRNA</td>
<td>Long noncoding ribonucleic acid</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LSPR</td>
<td>Localized surface plasmon resonance</td>
</tr>
<tr>
<td>M</td>
<td>Distal metastasis</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mPEG</td>
<td>methoxy polyethylene glycol</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MV</td>
<td>Microvesicles</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular bodies</td>
</tr>
</tbody>
</table>
MVE  Multivesicular endosome
mRNA  Messenger ribonucleic acid
miRNA  Micro ribonucleic acid
MUTEG  11-mercapto undecyl tetra (ethylene glycol)
MWCO  Molecular-weight cutoff
N  Regional lymph node
NA  Numerical Aperture
NaBH₄  Sodium borate hydride
NHS  N-Hydroxysuccinimide
NHS-PEG-SH  N-Hydroxysuccinimide-polyethylene glycol-thiol
NIR  Near-infrared
NP  Nanoparticle
nPES  Nanoplasmon-enhanced scattering
nPLEX  Nano-plasmonic exosome
NTA  Nanoparticle Tracking Analysis
nm  Nanometer
PE  Phycoerythrin
PEG  Polyethylene glycol
PES  Polyethersulfone
pg/mL  Picogram per milliliter
pM  Picomolar
PR  Progesterone receptor
PSMA  Prostate-specific membrane antigen
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rab</td>
<td>Ras-associated binding</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operation characteristic</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute Medium</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SAW</td>
<td>Surface acoustic wave</td>
</tr>
<tr>
<td>SEC</td>
<td>Size-exclusion Chromatography</td>
</tr>
<tr>
<td>SEDIA</td>
<td>Single exosome dual image analysis</td>
</tr>
<tr>
<td>SERS</td>
<td>Surface-enhanced Raman scattering</td>
</tr>
<tr>
<td>Si</td>
<td>Silicon</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor activating protein receptor</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SPRi</td>
<td>Surface plasmon resonance imaging</td>
</tr>
<tr>
<td>SVT</td>
<td>Single vesicle technologies</td>
</tr>
<tr>
<td>T</td>
<td>Primary tumor size</td>
</tr>
<tr>
<td>T-cell</td>
<td>T lymphocyte</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TMB</td>
<td>3′3′,5′5′-tetramethylbenzidine</td>
</tr>
<tr>
<td>TMN</td>
<td>Primary tumor size, regional lymph nodes, and Distant metastasis</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple-negative breast cancer</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TSG</td>
<td>Tumor susceptibility gene</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
</tr>
<tr>
<td>------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>TRPS</td>
<td>Tunable Resistive Pulse Sensing</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

1. Cancer

1.1. Cancer Statistics

Cancer is a disease characterized by uncontrolled growth and the spread of mutated cells, which can result in death when untreated. It is an assortment of more than 100 diseases in which a group of malignant cells defies the usual restrictions of cell growth and division, growing uncontrollably. These malignant cells will grow steadily, remaining within their original tissues, in-situ, or can invade nearby tissues and surrounding organs, becoming invasive. Finally, these abnormal cells shed signaling materials, such as exosomes, into the extracellular fluid, allowing the cancerous materials to travel to more distant areas of the body, causing metastasis. The development of cancer is not fully understood, nor the many factors that can promote cancer growth.

Cancer is one of the leading causes of death worldwide, resulting in nearly one in six deaths and the second most common cause of death in the United States. The four most predominant types of cancer leading to death in 2020 (~40% of all cancer cases in the world) were female breast cancer (~11.7%), lung cancer (~11.4%), colorectum cancer (~10.0%), and male prostate cancer (~7.3%). In 2020, there were ~19.3 million new cancer cases with a mortality rate of ~10 million worldwide. There were ~2.3 million new cases in the United States and ~0.6 million cancer deaths. The expected 5-year survival rate for all cancers combined in the United States is ~68%.

1.2. Stage Determination

Cancer stages are based on the degree of growth of the primary tumor and its potential to spread to other parts of the body. Cancer can be staged in many ways, such as clinical, pathological, post-therapy, and retreatment. Clinical staging is based on physical exams, imaging,
and biopsy results. The pathological stage is based on the results found during surgeries that give a better understanding of the margins and cellular information. After a patient's first treatment, post-therapy staging is implemented by measuring the disease’s responses. Finally, retreatment staging only happens if cancer recurs or progresses when the disease does not respond to treatment.\textsuperscript{13,14} Therefore, proper staging is crucial for determining prognosis as well as establishing optimal treatment.

Although there are many staging systems for cancer. Some cancers, such as leukemia, do not have staging systems, or testicular cancer has no stage IV.\textsuperscript{13} Tumor, Node, Metastasis (TNM) staging and the numbered group staging are primarily used in the clinical system to give a much more detailed diagnosis of the disease. In contrast, the more basic single-word staging system is used more predominantly by cancer registries.\textsuperscript{13,14}

1.3. Description of Cancer Stages

All types of cancers can be categorized using description staging. Cancer registries use this method more often than in the clinical setting. These descriptions use a five-category system with a simplified one-word explanation: \textit{In-situ}, Localized, Regional, Distant, and Unknown. \textit{In-situ} is defined as having abnormal cells that have not spread. Localized is defined as being limited to the location where the cancer started but having no signs of spreading to other locations. Regional is defined as cancer that has spread to nearby lymph nodes, tissues, or organs. Distant is defined as cancer having spread to other parts of the body. Unknown is defined as needing more information to be staged.\textsuperscript{14}

1.4. TNM Staging System

Another staging system, the TNM system, is predominately used in a clinical setting but cannot be used for all cancers. This system is based on primary tumor size (T), regional lymph
nodes (N), and distant metastasis (M). Six categories determine primary tumor size. TX, where the tumor is immeasurable. T0, where no tumor is found, and T1-4 is determined by the size of the primary tumor. The higher the number, the larger the tumor. Five categories determine the regional lymph node. NX, where the nearby lymph nodes cannot be measured, N0 has no cancer in the nearby lymph nodes, and N1-N3 is defined by the location and number of lymph nodes containing cancer. Finally, distant metastasis is determined by three categories. MX is defined as immeasurable metastasis, M0 when cancer has not metastasized, and M1 when cancer has metastasized.13,14

Other notes can be added: c for clinical staging, p for pathology staging, and y for restaging cancers.13 Pathologists can also grade cancer depending on what cells look like under a microscope. These can be defined in two methods depending on the cancer. The first grading system is categorized as low-grade- or high-grade- cancer, where cells look primarily normal and usually grow more slowly, or where cells look more abnormal, tending to grow more quickly, respectively. Grades (G) can also be defined in a system as G1-3. G1 is cancer cells that look normal and grow slowly, G2 is cancer cells that look abnormal and grow faster than healthy cells, and finally, G3 is cells that look abnormal and are growing or spreading more aggressively.13,15

1.5. Stage Grouping System

The stage grouping system is similar and can be used with the TNM system. This system is divided into five categories: stages 0, I, II, III, and IV. Stage 0 is defined as having abnormal cells that have not spread but can also be referred to as in-situ. Stages I-III can be grouped as cancer is present, with the higher the number, with the larger representing the extent to which the tumor has spread to surrounding tissues.14 However, these stages can be broken down into a more detailed explanation. Stages I and II are considered early stages. Stage I is localized to a small area and not
spreading, while stage II is defined as cancer having grown but not spreading. Stages III and IV are considered late stages. Stage III is defined as cancer that has grown larger with the possibility of spreading to the lymph nodes or other tissues. In contrast, Stage IV is classified as the tumor having metastasized to other organs and tissues.\textsuperscript{13}

Other factors can also affect cancer staging, such as cell type, tumor location, tumor marker blood levels, and laboratory testing.\textsuperscript{13} Cancers forming in specific body parts start in different variations of cells, affecting treatment, such as types of esophagus cancers. These cancers are usually squamous cells or adenocarcinomas, which are staged differently. The primary location of the tumor can also affect staging, such as esophageal cancer’s primary location being in the upper, middle, or lower region. In addition, the blood levels of specific tumor biomarkers can affect the stage of cancer, such as prostate-specific antigens from prostate cancer. Finally, laboratory tests on cancer cells, such as BC biopsies, are essential to staging.

2. Breast Cancer

2.1. Breast Cancer Statistics

In 2020, BC eclipsed lung cancer as the leading cancer diagnosis worldwide for women, with \textasciitilde2.3 million (11.7\%) of all new cases combined in both men and women.\textsuperscript{4} It is the fifth leading cause of cancer death worldwide, after lung, colorectum, liver, and stomach. The BC mortality rate worldwide is \textasciitilde0.7 million (6.9\%).\textsuperscript{5} As of 2023, it is estimated that BC will be the most diagnosed form of cancer, of all new cases, in both men and women in the United States, with \textasciitilde0.3 million or \textasciitilde15.3\% and \textasciitilde31.4\% of new cases in women alone. With an estimated mortality rate of \textasciitilde43,000, or \textasciitilde7.0\%, in both men and women and \textasciitilde15.0\% for women alone.\textsuperscript{8} The subtype, human epidermal growth factor receptor 2 (HER2)-positive BC, accounts for \textasciitilde14\% of all BC cases in the United States.\textsuperscript{9}
2.2. *Causes of Breast Cancer*

In BC, a genetic mutation of a single cell in the milk-producing ducts is usually the primary cause allowing for a growth advantage with the ability to spread uncontrollably. This genetic mutation frequently occurs in the breast cancer genes (BRCA1 and BRCA2) and is often seen as a genetic predisposition for BC. BRCA1 is located on chromosome arm 17q21 and is involved in cancer growth in up to 52% of high-risk patients. While BRCA2 can be found on chromosome arm 13q12-13 and is developed in up to 32% of high-risk patients.\(^\text{10,11}\) These genes can be found in the male and female genomes, but they can break out or remain dormant despite their presence. Therefore, BC does not have to emerge in each generation unless triggered by other factors.\(^\text{10,12}\)

Another significant cause of cell mutation is an imbalance of hormones. This mutation can be caused by several different factors, such as a woman’s first menstruation cycle, late menopause, first pregnancy after the age of 30, short lactation periods, and extensive use of estrogen supplements.\(^\text{16,17}\) During pregnancy, hormone production is increased to supply both the mother and fetus. This hormone production creates an abundance of hormones to support the creation of new life but also makes the perfect environment for tumor growth.\(^\text{18}\) Dietary habits are a significant determinant in oncogenesis, such as alcohol consumption, imbalance of fat-based diet in adolescence, and obesity, to name a few. It has also been reported that the intake of specific fatty acids, such as palmitic, stearic, and linoleic acids, is associated with increased risks of BC.\(^\text{19}\)

Another topic of discussion is the correlation between excessive stress and oncogenesis. Chiriac et al. reviewed the correlation between psychological stress and BC incidents.\(^\text{20}\) Using PubMed and Medline databases, 1813 articles from 1966 to 2016 were found using keywords. From the bulk articles found, 52 met their eligible criteria, having 26 articles identifying a link between BC and life stressors.\(^\text{20}\) The remaining articles did not find strong enough evidence or were unable to classify their findings. Finally, the probability of BC increases with age, doubling
every decade until menopause. BC is usually a more severe and aggressive form of the disease when it is found in younger women.\textsuperscript{21}

2.3. Different Types of Breast Cancer

BC can be categorized as invasive or non-invasive based on its relation to the basement membrane, or basal lamina, a thin dense layer of a specific extracellular matrix structure supporting epithelial and endothelial cells that surrounds muscle and fat cells.\textsuperscript{22,23} Non-invasive BC can also be subdivided into groups: lobular carcinoma in situ, which develops from atypical lobular hyperplasia, and ductal carcinoma in situ develops in the mammary ducts.

The most commonly accepted classification of BC is the immunohistochemical viewpoint. This classification is established on the biomarker expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor (HER).\textsuperscript{24} However, BC can also be molecularly typed into six categories, Luminal A, Luminal B, Basal-like, Triple-negative, HER2-positive, and Claudin low BC.\textsuperscript{25} Luminal A BC is an ER-positive, HER2-negative, high-grade PR, and low-grade grade Ki-67. Luminal B BC is an ER-positive, HER2-negative, with either low-grade PR or high-grade Ki-67. Basal-like BC usually lacks expression of molecular targets that respond to aromatase inhibitors or trastuzumab. Triple-negative BC is ER-, PR-, and HER2-negative. Claudin low is typically a triple-negative BC but also has a low epithelial cadherin expression.

2.4. Significance of Early Breast Cancer Detection

Early detection is one of the most crucial challenges in cancer survival. Early diagnosis increases treatment possibilities and reduces mortality risk. Unfortunately, ~12.9\% of women in the United States will be diagnosed with BC within her lifetime.\textsuperscript{26} The overall five-year relative survival rate for women diagnosed with localized BC is ~99.1\%. In contrast, the survival rate for
regional BC is ~86.1%, and for distant BC, it is ~30.0%.\textsuperscript{27} Women are encouraged to develop a personalized screening plan based on family history, risk assessment, genetic dispositions, or personal preferences. Standard screening for BC is a mammogram that can detect cancer up to two years before the tumor can be felt by breast examination. Women over the age of 40 are recommended to receive a yearly mammogram, and women at high risk are recommended for breast magnetic resonance imaging in addition to an annual mammogram.\textsuperscript{3,28} There are still many false negative diagnoses using these methods, and thus there is an urgent need to develop more accurate determinations for the early detection of BC.

3. Exosomes

3.1. History of the Exosomes

The origin story of the “discovery” of the exosomes is conflicted among the research community. In 1981, Trams et al. described a secondary population of vesicles of approximately 40 nm when examining shed vesicles.\textsuperscript{29} In 1983, Pan et al. described vesicles secreted by reticulocytes during maturation,\textsuperscript{30} and Harding et al. reported a receptor-bound vesicle dissociated from the cell surface.\textsuperscript{31} Exosomes were initially thought to be a function for the cell to discard/recycle redundant material that was not needed.\textsuperscript{32,33} The designation of “exosome” was not coined until 1987 by Johnstone et al.\textsuperscript{34–38} Exosomes are currently defined as heterogeneous membrane-bound extracellular vesicles (EV)s with a 40-200 nm diameter and a density of 1.10-1.18 g/mL.\textsuperscript{39,40}
3.2. Exosomes Morphology, Biogenesis, and Composition

3.2.1. Morphology

Exosomes are nanosized heterogeneous membrane-derived EVs containing a phospholipid bilayer released by exocytosis by various cells. EVs are commonly referred to as exosomes, microvesicles, apoptotic bodies, ectosomes, shedding vesicles, and microparticles, among other names. EVs are produced by outward budding and the cell’s lipid membrane fission. EVs range in size from 40 nm to 1µm, membrane-bound vesicles released from cells that can transport cargo for intercellular communication. Based on biogenesis or release pathways, EVs are characterized as microvesicles (MVs), exosomes, oncosomes, and apoptotic bodies. Small ectosomes are secreted by direct outward budding of the plasma membrane, which can be confused with exosomes.

Exosomes are round or cup-shaped, possibly due to dehydration, and range from 40 to 200 nm. Due to different purification and enrichment methods, the size and shape do not have an exact definition. In addition, exosomes are stable upon repeated freezing and thawing but will have some degradation. With past and current research, the definition of the exosome has and will continue to grow and conform to a more solid understanding.

3.2.2. Biogenesis

Exosome biogenesis and its shedding into the extracellular space is a faceted and complex path originating with early endosomes. Endosomes are organelles that primarily contribute to the sorting and delivering of intracellular material within a eukaryotic cell. Initially, endocytic vesicles are formed through inward budding of the plasma membrane, followed by multiple endocytic vesicles fusing to form early endosomes and multivesicular endosomes (MVE), as seen in Figure 1.1. An early endosome undergoes a maturation process characterized by acidification
and protein content changes. The shape and intracellular location changes determine the development of early to late endosomes. Early endosomes are located near the cellular membrane with a tubular shape, while late endosomes are located near the nucleus with a round shape. The early endosomal maturation can also be detected with the formation of intraluminal vesicles (ILVs) membrane budding, allowing for cytosol-containing proteins, nucleic acids, and other macromolecules to be incorporated into newly formed ILVs. This subgroup of late endosomes is known as multivesicular bodies (MVBs).

Figure 1.1: Biogenesis of MVs and exosomes. MVs bud directly from the plasma membrane, whereas exosomes are represented by small vesicles of different sizes formed as the ILV by budding into early endosomes and MVEs and are released by the fusion of MVEs with the plasma membrane. Other MVEs fuse with lysosomes. The point of divergence between these types of MVEs is drawn at early endosomes, but the existence of distinct early endosomes feeding into these two pathways cannot be excluded. Red spots symbolize Clathrin associated with vesicles at the plasma membrane (clathrin-coated vesicles [CCV]) or bilayered Clathrin coats of endosomes. Membrane-associated and transmembrane proteins on vesicles are represented as triangles and rectangles, respectively. Arrows represent proposed protein and lipid transport directions between organelles and between MVEs and the plasma membrane for exosome secretion. Reprinted with permission from Raposo, G.; Stoorvogel, W. (2013) Copyright 2013, Rockefeller University Press.

MVBs have two possible fates depending on regulatory mechanisms that are not fully understood. One proposed mechanism is that the MVB outcome depends on the cholesterol (CLS)
content. An MVB containing a higher CLS composition can fuse with the cellular membrane, causing the subsequent secretion of ILVs into the extracellular region. These ILVs entering the extracellular region are known as exosomes. If the CLS content of the MVB is low, a lysosome will undergo fusion with the MVB, and its contents will be degraded. The second proposed mechanism construes ISGylation of MVB proteins, promoting lysosome fusion and inhibiting exosomal release. Fusion of the MVBs with the cellular membrane is enabled, but not limited to Rab genes, GTPases, and SNARE proteins.

3.2.3. Composition

The molecular contents of exosomes reflect their parent cells. Exosomes comprise a lipid bilayer containing membrane proteins, cytosolic, nuclear proteins, extracellular matrix proteins, metabolites, nucleic acids, mRNA, noncoding RNA, and DNA (Figure 1.2). Though not all exosomes are built the same, their inter- and external physiological composition reflect the donor cells. The exterior is predominately made of the lipid bilayer from the donor cell, but the mechanism for forming their inner makings is not fully understood. Figure 1.2 is a diagram of the fundamental structure of an exosome.

Exosomes contain, but are not limited to, Rab proteins (essential in the regulation of membrane trafficking intracellular transport among many others), annexins (calcium and phospholipid binding proteins for membrane trafficking and fusion), tubulin, actin, actin-binding proteins, lipids (such as lysobisphosphatidic acid), and various metabolic enzymes. It also includes heat shock proteins (HSP), the molecular chaperones that bind to polypeptide chains to prevent protein misfolding, such as HSP60, HSP70, HSP90, HSPA5, and CCT2. Additionally, it contains multiple types of nucleic acids such as dsDNA, mRNA, miRNA, tRNA, lncRNA, and viral RNA.
Figure 1.2: The molecular components of exosomes. Exosomes are known as phospholipid bilayer (colored blue) enclosed vesicles containing various proteins on their membrane. Internally, their cargo can be comprised of nucleic acids and numerous proteins.\textsuperscript{56} Reprinted with permission from Whitehead, C.A.; Luwor, R.B. (2012) Copyright 2012, Nancy International LTD Subsidiary AME Publishing Company.

Exosomes also contain lipids like CLS, sphingomyelin, hexosylceramides, phosphatidylserine, and saturated fatty acids.\textsuperscript{32} Membrane proteins include tetraspanins (a family of more than 30 proteins) such as CD9, CD37, CD53, CD63, and CD81, Alix, and TSG101, integrins, epithelial cell adhesion molecules (EpCAM), HER and major histocompatibility complexes I and II.\textsuperscript{32,37,56,60} Exosomes hold incredible clinical potential as they carry the molecular constituents of their parental cells, and therefore disease markers from cells can be probed through exosomes.\textsuperscript{33,48,63,64}

3.3. Exosome Isolation and Purification

High-purity exosomes are needed to study the properties and functions of exosomes accurately. The isolation and purification process allows the differentiation of specific properties such as size, morphology, buoyancy, and protein composition. The three major methods that are used to isolate/purify exosomes are differential centrifugation/ultracentrifugation, size-exclusion
chromatography, and immunoaffinity isolation. The ability to successfully separate exosomes from the other EVs and debris found in plasma and cell culture mediums is essential for the progression of liquid biopsies.

3.3.1. Centrifugation/ultracentrifugation

Differential centrifugation is currently the gold standard for exosome isolation, with the ability to separate cells, exosomes, proteins, etc., based on size. Media usually goes through a step process to eliminate larger to smaller contents. The first step is centrifugation at 300g for 10 minutes to eliminate cell debris, and multiple steps of 1000g, followed by 20,000g to remove larger vesicles. Finally, ultracentrifugation is used with speeds greater than (> 100,000g to purify exosomes from protein contaminants. The downside to this process is that it takes approximately five hours or more. Furthermore, differential centrifugation can often result in a lower exosome recovery with co-precipitated protein aggregates contams.

3.3.2. Size-exclusion chromatography

Size-exclusion chromatography (SEC) can separate exosomes from protein aggregates but is usually applied after centrifugation to remove aggregates. Larger particles, such as exosomes, elute faster than smaller analytes, such as proteins, thus isolating by collecting the eluted fraction at a specific time. Muller et al. reported that SEC should be applied before ultracentrifugation to help remove excess plasma protein and other smaller molecules. They compared plasma-derived exosome fractions that had and had not been processed with SEC using transmission electron microscopy (TEM). It was reported by Baranyai et al. that exosome purity can depend on the matrix and is not always as efficient as ultracentrifugation methods alone.
3.3.3. Immunoaffinity isolation

There are multiple methods for immunoaffinity isolation, such as chips, beads, or a plate. Immunocapture assays capture exosomes on their immunophenotype using a monoclonal antibody (Ab) directed against an antigen exposed on the targeted exosomes.\(^4\) A common immunoaffinity approach employs Ab-coated magnetic beads to capture exosomes containing target-specific biomarkers in bodily fluids. This method allows for the isolation of specific exosomal subpopulations but is not suited for large biological samples for exosome isolation.\(^6\)

3.4. Exosome Characterization

Another challenge in properly comparing the properties and functions of exosomes from other secreted membrane vesicles and aggregates is characterization. However, these challenges can be overcome by finding the appropriate size distribution and concentrations of exosomes. Three approaches used to accomplish this undertaking are nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), and tunable resistive pulse sensing (TRPS).\(^7\)–\(^7\) The ability to successfully characterize exosomes from the other secreted vesicles and debris found in plasma and cell culture mediums is essential for the progression of liquid biopsies.

3.4.1. Nanoparticle tracking analysis

The first and one of the most common methods for the characterization of exosomes is NTA. This technique analyses particles in a liquid using video analysis. Particles are illuminated by a light source, in this case, a laser. The scattered light from the particles is recorded using a Charge-Coupled Device (CCD) or Complementary Metal Oxide Semiconductor camera. The video of the particles is analyzed utilizing Brownian motion. The provided results give both concentrations of particles/mL and particle sizes ranging from 10 to 1000 nm. This instrument can
also be used in a fluorescent method, providing the ability to analyze particles prelabeled with fluorophores.\textsuperscript{72,75}

3.4.2. \textit{Dynamic light scattering}

A second method for exosome analysis is DLS. DLS works on similar concepts as NTA but only determines the size, not the concentration. This method provides the hydrodynamic (HD) size distribution of particles based on the fluctuating scattered light intensity after irradiation from the light source. The particle’s size is calculated using the transformation between fluctuation rates and diffusivities of the particles over time.\textsuperscript{76} The higher intensity of the scattered light, the larger the particles.

3.4.3. \textit{Tunable resistive pulse sensing}

A third method, newer technique, for exosome quantification is TRPS. This method can measure both the size distribution and concentration.\textsuperscript{77} Particles are suspended in an electrolyte solution that measures the voltage differences generated by the analyzed particles. A voltage pulse is generated each time a particle passes through the nanopore. Each pulse measured is then fitted into a calibration standard, which subsequentially gives the size distribution and concentration of the exosome.\textsuperscript{73} TRPS offers greater sensitivity and accuracy, but it is susceptible to system stability issues, such as nanopore blockage, sensitivity issues, and particle size being too small to detect against background noise.\textsuperscript{77}

4. \textbf{Roles of Exosomes in Cancer and Breast Cancer}

In cancer, exosomes fulfill a significant role in tumor initiation and growth. Furthermore, they perform essential functions in the malignant progression of cancer. These particular mechanisms are immunosuppression through the inhibition of immune cell proliferation, induction of apoptosis-activated cytotoxic T-cells, and suppression of natural killer cell activity, giving the
mutated cell a growth advantage. The specific cause of the mutation is unclear, but the perpetrators responsible demonstrate significant roles in oncogenesis, including genetic predisposition, alcohol consumption, excess stress, hormonal disbalance, and diet.32

With a rising research interest in the field of cancer, new findings are becoming more subsequent that exosomes play an essential role in cancer development (Figure 1.3)78. Playing roles in cell-to-cell communication, horizontal transfer of genetic material, induction or inhibition of protein translation, disposal of cellular waste, and regulation of immune responses. Exosomes are secreted by all types of cells, including cancer cells. Therefore, the exact mechanisms to sustain the body are used against it with such diseases.

**Figure 1.3: The role of exosomes in breast cancer.** Exosomes are released from breast cancer and stromal/cancer-associated fibroblast cells into the extracellular milieu and tumor microenvironment.78 Reprinted with permission from Lowry, M.C.; Gallagher, W.M. (2015) Copyright 2015, Oxford University Press. *Invasion and Metastasis.*

The role of the exosome in BC can cause the invasion and metastasis of normal tissue, as seen in all metastatic processes. Reports show the involvement from the initial stages of the primary tumor cell's localized invasion of nearby tissues to reshaping of the extracellular matrix
for impending tumorigenesis, acquisition of migration capacity, and distant spread to the most common metastasis sites (bone, brain, liver, or lung).\textsuperscript{79} Exosomal roles in metastasis were first described in the triple-negative breast cancer (TNBC) cell line Hs578T. O’Brien et al. describe an exosomal transfer of phenotypical traits, causing increased proliferation, migration, and invasion to secondary BC cells (SK-BR-3, MDA-MB-231, and hepatic cell carcinoma (HCC) 1954). When TNBC exosomes were introduced to healthy cells, angiogenesis in human endothelial cells increased.\textsuperscript{80} Other studies have also shown findings such as exosome derived from TNBC causing enrichment of the enzymes responsible for the degradation of extracellular matrix, MDA-MB-231 derived exosomes having a presence for the migration promoting protein Caveolin-1, and HER2-positive BC patients having specific exosomal miRNA.\textsuperscript{81}

4.1. Immune System

Tumor cells have established various mechanisms to suppress immune system defenses to prevent cancer development, with exosomes being one of the primary mechanisms. Reports have shown that exosomes have positive and negative effects on immune responses.\textsuperscript{82} Tumor-derived exosomes can influence the immune system, promoting tumorigenesis in numerous methods. Suppression of, upregulation of regulatory, and apoptosis of T cells, as well as suppression of natural killer cells.\textsuperscript{83,84} It was reported that exosomes isolated from BC cell lines MDA-MB-231 and BT-474 caused intense immunosuppression activity, negatively modifying T-cell proliferation through TGF-β.\textsuperscript{85} Furthermore, Jang et al. used exosomes isolated from murine BC cells to show inhibition of macrophage activity after the transport of miRNA miR-16 into tumor-associated macrophages.\textsuperscript{86} Finally, it was concluded by Gomes et al. that exosomes isolated from the MDA-MB-231 cell line can induce platelet aggregation. This is a vital process for circulating tumor cells to remain undetected by the immune system.\textsuperscript{87}
4.2. Apoptosis

Apoptosis is physiologically-programmed cell death and is a crucial mechanism for maintaining a healthy cell population in multicellular organisms.\textsuperscript{88} This device is critical for tumor cells to evade apoptosis and allows the ability for the cell to thrive, making it understood that exosomes participate in this restriction process. It was reported by Shi et al. that exosomes isolated from 4T1 BC cells suppressed apoptosis in tumor cells such as CD133+ stem cells. This research group was able to show exosomal inhibition of apoptosis as well as the ability to enable proliferation.\textsuperscript{89} Another group, Wang et al., reported that long non-coding RNA H19 in exosomes prevented apoptosis. They showed that the overexpression of H19 enhanced resistance to doxorubicin-resistant BC.\textsuperscript{90}

4.3. Drug Resistance

Cancer treatment success rates highly depend on the therapeutic responses by overcoming cellular defenses for self-preservation of the affected target areas. In 1983, Harding et al. first described the shedding vesicle as a way cells eliminated waste and extra material from the cytosol into the extracellular space.\textsuperscript{31} This concept complicates novel therapeutics, disposing of potentially highly effective therapies from the cell before they can help.\textsuperscript{91,92} Tumor-derived exosomes promote drug resistance in BC by triggering changes in cellular homeostasis by increasing DNA damage repair, creating a bypass in signaling pathways, and transferring functional cargo to upregulate ER\textsubscript{α} expression and hormone-independent signaling.\textsuperscript{93,94} The overexpression of HER2 in BC patients has produced strong evidence of increased tumor aggression.\textsuperscript{95} It has been shown in novel HER2 targeting therapeutics that the efficiency of the initial response diminishes significantly within the first year.\textsuperscript{96} Tumor cells release exosomes with overexpression of HER2 to areas affected by the therapeutic, reducing the drug’s effectiveness.
5. Current Detection Methods for Exosomes

5.1. Exosomes in Liquid Biopsy

Exosome-based liquid biopsies have the potential for diagnosis, monitoring progression, and therapeutic responses to cancer as well as other diseases by way of their biomarkers. Exosomes are present in all body fluids, allowing the ability to obtain samples effortlessly without invasive surgeries and permitting real-time monitoring. With this availability, exosome isolation is more convenient than isolating circulating tumor cells. Exosomes are extremely stable vesicles allowing for both short-term storage at 4 °C (no more than ten days) and long-term storage at -70 °C or lower, due to their stability. This durability can also reduce shipping costs if exosomes need to be transferred to other facilities.

Exosomes comprise a wide variety of surface proteins, with a few being considered exosome specific such as CD81, Alix, or TS101, allowing the ability to be easily separated and analyzed. Exosomes are emerging to be exceptional biomarkers capable of accurately determining and diagnosing diseases compared to other serum-based biomarkers. Exosome detection is not limited to distinguishing surface biomarkers. Their interior molecular content can also provide much-needed information as well. Exosomes contain genetic material such as DNA, mRNA, miRNA, and more, that can offer more information representative of the donor cell than the cell-free DNA of dying cells. In 2016, the first commercial-based exosome test became available for prostate cancer. This exosome-derived RNA biomarker test is recommended by the National Comprehensive Cancer Network and has been used by more than 50,000 patients in their decision-making process.
5.2. Protein Analysis Methods

The donor cell primarily affects an exosome’s protein composition, making detection-specific methods for extra- and inter-exosomal analysis essential for detecting and diagnosing cancer. There are > 75 current studies that have identified > 2,300 exosome-specific proteins. These studies show excellent possibilities for developing early cancer detection using protein markers.\(^{103}\) The most conventional methods for protein analysis are Western Blot, ELISA, mass spectrometry (MS), and flow cytometry (FC).\(^{104}\) However, there are some challenges with these techniques for exosome detection or clinical uses. They are difficult and expensive to isolate due to their heterogeneity, requiring significant experience for complex pre-processing, very time-consuming, use large sample volumes, and have a low refractive index.\(^{105,106}\) New advances will bring the ability to simplify and improve current methods, develop new technologies for analysis using exosome detection, and implement scientific research into standardized clinical applications.

5.2.1. Western blotting

Western blotting, also known as immunoblotting, is a widely recognized technique generally used for assessing the presence and integrity of proteins in exosomes.\(^{107-117}\) This method involves gel electrophoresis, membrane blotting, and Ab probing.\(^{118}\) This technique is immeasurably valuable because the proteins are separated based on their physical properties, such as molecular weight and charge, allowing them to be categorized into different sizes. However, the downside to this method is that it is incredibly time-consuming, requiring up to 24 h, and due to lysing, whole vesicles cannot be analyzed. Using Western Blotting and atomic force microscopy, Maji et al. analyzed the functions of the exosomal protein Annexin II (exo-Anx II), one of the most prevalent exosomal proteins.\(^{119}\) Their research aimed to depict the specific functions of exo-Anx II in the development and metastasis of BC using MCF10CA1 and MDA-
MB-231 BC cell lines and in vivo animal models. They showed that exo-Anx II expression was approximately five times higher in aggressive BC types than in normal and premetastatic cell lines. As for the in vivo study, exo-Anx II demonstrated roles in cell transformation favoring metastasis. Their findings stated that the exo-Anx II function promotes angiogenesis and metastasis.

5.2.2. Enzyme-linked immunosorbent assay

Currently, ELISA is a benchmark method for protein analysis. It is cost-effective, has reproducible results, and is highly sensitive, with quantifiable data. However, this technique is extremely time- and labor-intensive, requiring relatively large sample and reagent volumes. Additionally, most ELISA protocols depend on an enzyme-mediated signal amplifier to achieve the appropriate sensitivity, causing a linear variation in the results. To date, ELISA has been the primary method for analyte detection in biological samples in scientific research and clinical settings. Therefore, this method gives an evident and essential need for developing applications in exosomal protein analysis. Moon et al. used ELISA to provide further evidence in determining an increased fibronectin (FN) expression as a significant protein biomarker on circulating exosomes in BC patients for early detection. Blood samples were obtained from healthy donors, BC patients (stages 0-IV), patients post-operation, patients with benign tumors, and patients with non-cancer diseases. Their results showed significantly higher FN values in the BC cohort than all others. It was further reported that FN could be used as a potential marker for early BC detection due to the independent elevation levels between BC subtypes.

5.2.3. Mass spectrometry

Protein analysis studies using MS-based analytical methods offer high sensitivity, accuracy, and resolution for heterogeneous samples. Mass spectrometry is time-consuming, requires significant analyst expertise, has limitations and disadvantages associated with coupling
methods, and does not allow for complete vesicle analysis.\textsuperscript{104,132} Exosomes must be fractionalized into peptide segments, commonly performed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis, widely performed by one of these methods, two-dimensional (2D) liquid chromatography, or isoelectric-focusing based fractionation.\textsuperscript{104} MS-based profiling permits extensive identification and quantification of exosome-derived proteins, allowing for more significant contributions to creating data libraries.\textsuperscript{41,133–136} Risha et al. explored the differences in protein compositions between exosomes derived from metastatic BC MDA-MB-231 and normal epithelial cell MCF10A, trying to identify potential new protein markers for early detection.\textsuperscript{137} Nano-liquid chromatography coupled with tandem MS was utilized for this analysis method. They identified 726 unique proteins in MDA-MB-231 out of 1,107 proteins in both exosomal-derived cell lines. Furthermore, 87 proteins identified were considered to have been associated with cancer, and 16 of the proteins were further classified to be significant for metastasis. Finally, membrane/surface protein markers, glucose transporter 1, glypican 1, disintegrin, and metalloproteinase were identified as potentially unique to BC biomarkers.

5.2.4. \textit{Flow cytometry}

Flow cytometers are expensive instruments and, in their conventional forms, are not constructed to analyze particles below approximately 300-500 nm.\textsuperscript{104,138,139} However, it is still a preferred method for exosomal protein analysis for many researchers.\textsuperscript{140–148} Flow cytometry provides extremely accurate quantitative information that can facilitate subpopulation analysis, making it the most suited for diagnostic and clinical research compared to the methods mentioned earlier.\textsuperscript{149} Exosome analysis is complicated due to its size compared to cell analysis because exosomes have fewer antigens on their surface, giving a lower emitted fluorescence from each vesicle and having a higher background signal with the limitations in post-stain washing.\textsuperscript{150} Many
researchers are confronted with the problem of increased optical backgrounds masking the presence of small vesicles and false-positive signals produced by immunoglobulin aggregates.\textsuperscript{104}

New applications have been developed for FC, such as using micrometer-sized latex beads for multivesicular binding or constructing selective FC instruments that can distinguish nanoparticles smaller than 100 nm. Kibria et al. were able to demonstrate exosome detection at the single vesicle level with the use of a highly sensitive FC.\textsuperscript{151} Utilizing an improved, rapid analytical approach for micro FC, they were capable of quantifying the expression of two targeted proteins, CD44 and CD47, on the surface of exosomes derived from cell medium and blood plasma from healthy donors and BC patients. CD44 is linked with tumorigenesis and tumor progression, whereas CD47 is known to inhibit the ability of macrophages and natural killers to recognize a diseased cell. The authors also used CD47 expression to differentiate between healthy and cancerous populations but could not detect a statistical difference between the two populations using the protein marker CD44.

Many groups use modified FC methods as a preferred choice for SVT when using modified high-resolution methods coupled with amplified fluoresce labeling.\textsuperscript{138,139,143,144,152,153} Morales-Kastresana et al. attempted to address these limitations using nanoscale FC with fluorescent polystyrene beads.\textsuperscript{70} This group cultured immature dendritic cells using serial ultracentrifugation to isolate and concentrate exosomes from the medium, confirmed by NTA. The exosomes were stained using the carboxyfluorescein succinimidyl ester (CFSE), a fluorescent dye, then washed to remove an unlabeled dye using SEC. The CFSE-labeled exosomes were eluted, using fractions three and four for analysis. This method has advantages and disadvantages, such as using SEC helped to reduce background noise, but it is size-specific, allowing for some exosomes to not be observed. Although this method was successful, it would be highly cumbersome in the clinical
setting. With more developments in the future for FC, there is an unforeseeable potential for exosome research and early detection of diseases.

5.2.5. Miscellaneous techniques

There have been many novel and unique techniques for detecting the molecular content of exosomes reported over the past years. Many researchers have expanded their efforts on merging ideologies of advanced physical detection methods with immuno-based exosome capture and immuno-labeling probes due to the need for more sensitivity of current standard methods. Exosome detection and characterization have changed from the typical application of the lab-on-chip design,\textsuperscript{154,155}, attempting to merge the aspects of conventional approaches, using small sample volumes and minimal preprocessing. Lee et al. constructed a microfluidic device to immobilize exosomes in a neutravidin-coated microfluidic chamber for on-chip immune staining and fluorescence imaging.\textsuperscript{156} Their method used biotinylated exosomes captured on the surface of the device, then adding three different target protein fluorochromes for labeling. After labeling and detecting the first target protein labeled fluorochrome, the signal was quenched before adding the second label. This method allowed them to simultaneously detect three different proteins, potentially increasing the number of detection proteins using more fluorochromes. Furthermore, newly developing strategies are founded on the ideology of using surface plasmon resonance (SPR),\textsuperscript{157–162} fluorescence techniques,\textsuperscript{156,163–167} interferometric imaging,\textsuperscript{168} Electrochemical sensings,\textsuperscript{169–174} colorimetric,\textsuperscript{47,175,176} Micro-Nuclear Magnetic Resonance (\(\mu\)NMR),\textsuperscript{106,177,178} and surface-enhanced Raman scattering (SERS).\textsuperscript{179–181}

6. Overview of Chapter Contents

Chapter 2 will review AuNP-based technologies for exosome molecular detection and analysis, leading to a continued effort to create innovative nanomaterials to detect and characterize
exosomes. This chapter will summarize and include a discussion of plasmonic nanotechnology-based devices and methods developed in past decades to detect and analyze molecular exosomes intended for cancer exploration. The functional properties will be briefly defined for the nanomaterial described, followed by the application mechanism for exosome biomarker detection, profiling, and analysis. Reprinted (adapted) from {Ref 182}

Chapter 3, we will review the development and optimization of our DISVT methodologies for single exosome protein profiling. Intending to reduce sample volumes and preprocessing necessary for liquid biopsies requires unique and innovative devices designed to promote the use of nanomaterials for biomarker detection and quantification. In this chapter, we aimed to create a novel, simple, reproducible, and low-cost method for the capture and detection of exosomes using DF- (target) and fluorescent-(mask) microscopy, as well as a basic outline of our Single Exosome Dual Imaging Analysis (SEDIA) program to quantify single cancer-specific exosomes in a heterogeneous sample. Our assay’s high sensitivity and specificity allow us to identify a limited exosome population mixed with exosomes of various origins. Reprinted (adapted) with permission from {Ref 183}. Copyright {2023} American Chemical Society.

Chapter 4 will review the characterization of cell-derived cancer exosomes using DISVT. This method used our DISVT to label and detect exosomes derived from three BC cell lines, SK-BR-3, MDA-MB-231, and MCF-7, using the AuNPs conjugated with specific protein biomarkers HER2, EpCAM, and CD44. Again, the optimal methods developed from chapter two were used. Exosomes were first captured using the CD81 exosomal protein on a gold (Au) surface, labeled with target-specific proteins decorated on AuNPs, and finally localized using a fluorescent dye. Reprinted (adapted) with permission from {Ref 183}. Copyright {2023} American Chemical Society.
Chapter 5, we will review the evaluation of the potential of exosomes for early cancer detection using HER2-positive BC as the disease model using DISVT. With its high sensitivity and specificity, this method can identify explicit exosomal populations within the heterogeneous mixture. This technology is possible for early-stage cancer detection because cancer-derived exosomes are naturally mixed into many complex body fluids, masked by the dominantly amalgamate population of normal cell exosomes. To examine the potential of DISVT for early cancer detection, we profiled the HER2 expression on the plasma exosomes from HER2-positive BC patients at early stages and the locally advanced stage, HER2-negative stage III patients, and healthy donors. Exosomes are first immobilized onto a Au substrate with CD81 exosomal protein. Next, they are tagged with AuNPs conjugated with target-specific protein and localized using a Cyanine-5 (Cy5) fluorescent dye.\textsuperscript{183} Reprinted (adapted) with permission from \{Ref 183\}. Copyright \{2023\} American Chemical Society.
CHAPTER 2: REVIEW OF CURRENT GOLD NANOPARTICLE-BASED TECHNOLOGIES FOR MOLECULAR DETECTION AND ANALYSIS OF EXTRACELLULAR VESICLES

1. Introduction

Tissue biopsy is the benchmark clinical method for most cancer diagnostics, playing a vital role in establishing an optimal treatment plan for that individual patient. Nevertheless, tissue biopsies are invasive, costly, impractical for repeated testing, and unattainable for certain types of cancer. Additionally, tumors are heterogeneous and progress over time, making biopsy collection from the restricted tumor regions biased and can mislead clinical decisions. After surgery, the primary tumor and its margins are not accessible, inhibiting real-time treatment monitoring. Due to these limitations of tissue biopsies, the development of liquid biopsies, a means to detect and analyze cancer biomarkers in body fluids such as blood and urine, has become a driving factor in developing better cancer diagnosis and monitoring.

Currently, liquid biopsy uses emerging novel analytes such as EVs, including exosomes and MVs, from the biofluids for diagnostics, prognosis, and treatment monitoring of cancer and other diseases due to their unique features, such as protein, nucleic acid, or tumor cells. Exosomes are membrane-bound vesicles, continuously released into the extracellular matrix by nearly every cell, with exosomes of endosomal origins and microvesicles of the plasma membrane. They can carry DNA, various RNAs, and numerous proteins, such as tetraspanins, receptors, and adhesion molecules, reflective of their donor cell. Exosomes derived from tumor cells can transfer oncogenic factors through intercellular communication, causing the regulations of angiogenesis, immunity, and metastasis to promote tumorigenesis and progression. Tumor-derived exosomes enter the blood and many other body fluids such as
urine, saliva, ascites, and cerebrospinal fluid giving exosomes the ability to be a robust and novel source of biomarkers for noninvasive liquid biology in scientific and clinical medicine.

Exosomes are commonly collected and isolated from body fluids using density separation methods (differential centrifugation and precipitation), size- and affinity-based methods, and microfluidics using one or more techniques. These applications eliminate protein and cell debris, capture a specific molecular exosome subtype for downstream analysis, and concentrate the exosomes to facilitate subsequent analysis.

Figure 2.1: Overview of the application of nanomaterials and detection methods for extracellular vesicles. Abbreviation: NPs-nanoparticles, SPR-surface plasmon resonance, SERS-surface enhanced Raman scattering, μNMR: micro nuclear magnetic resonance.

Dynamic light scattering, NTA, and electron microscopy can readily characterize size and morphology for exosome molecular detection and analysis. However, there are challenges due to the relatively small sizes of exosomes, proportionally lower numbers of analytes per exosome, down to a single analyte for smaller exosomes. Therefore, various techniques have been used for
characterization, including but not limited to traditional protein and nucleic acid detection methods such as polymerase chain reaction, ELISA, western blot, FC, MS, and emerging methods using advanced methodologies, materials, and devices.\textsuperscript{130,199} Compared to traditional approaches, advanced techniques can enhance exosomes molecular detection and analysis concerning simplicity, sensitivity, and efficiency, thus leading to a promising future in the clinical setting.

Among the growing molecular characterization techniques, nanotechnology is vital in the unique structural, and functional properties of nanoscale materials.\textsuperscript{200–205} Nanomaterials exhibit extraordinary functional properties, generally not available from lysed molecules and bulk materials. Nanostructures have a large surface-to-volume ratio allowing for high efficiency of target interaction. The structural and functional properties can be utilized in developing new assays to overcome the limitations of traditional techniques. Figure 2.1 shows how exosome molecular detection has a variety of nanomaterials involved. Im et al. developed a nano-plasmonic exosome (nPLEX) assay based on SPR sensing using periodic nanohole to detect surface protein markers on exosomes, having a higher sensitivity of $10^4$-fold than western blot and $10^6$-fold than ELISA.\textsuperscript{160} Liang et al. developed a different nanoplasmonic approach to quantify tumor-derived exosomes in plasma samples on a microliter scale using the SPR light scattering properties of spherical and rod-shaped Au nanoparticles (NPs).\textsuperscript{206} Kwizera et al. reported a SERS method employing gold nanorods (AuNRs), establishing a limit of detection (LOD) of $2 \times 10^6$ exosomes/mL.\textsuperscript{179}

Surface plasmon resonance occurs when electrons are transferred at the interface of two materials. The physical part of the dielectric function changes signs across the interface when stimulated by incident light, causing a resonant oscillation of those electrons. The generated surface plasmon polariton is a non-radiative electromagnetic wave that propagates on the dielectric interface, which is sensitive to small changes on this boundary, such as the absorption or
disassociation of molecules or small biological entities such as proteins. This characteristic has made SPR an attractive technology for researchers interested in molecular and biological sensing, including detecting exosomes and MVs.

When the plasmonic materials’ dimension decreases to the nanoscale, SPR is limited to the surface of the nanomaterials and becomes a more robust form of SPR called localized surface plasmon resonance (LSPR), obtained through either patterned nanostructure on a 2D substrate or colloidal NPs of novel metals such as Au and silver (Ag). For decades, colloidal AuNPs appealing LSPR-enhanced optical properties have been an attractive nanomaterial across many fields, from material science to biomedical applications. LSPR is size, shape, and structure sensitive, with the ability to tunable optical properties from visible to near-infrared (NIR) regions. For example, when the shape of the AuNP is changed from a solid sphere to a rod, the LSPR is red-shifted into the NIR region, giving it the ability to penetrate tissue, suitable for in vivo photothermal cancer therapy. Due to LSPR, the AuNP absorption and light scattering orders of magnitude are more substantial than that of non-metallic NPs and individual organic dyes. Due to their intense light scattering properties under DF microscopy, AuNPs appear as bright fluorescence analogs. Unlike fluorophores, AuNPs do not photo-bleach, allowing them to be used as excellent contrast agents for bioimaging. Furthermore, LSPR induces a strong electromagnetic field around the NPs, enhancing the Raman scattering signals of adsorbed molecules. SERS spectroscopy offers the detection and quantification of the targeted molecules based on their fingerprint. The following sections will discuss different applications of plasmonic NPs for exosome molecular detection and analysis based on the mechanisms of signal readout.
2. Surface Plasmon Resonance Sensing

Traditional SPR sensing is generally accomplished using Au-coated SPR sensor chips. Typically, the chip is directly functionalized with detection Abs to recognize target-specific surface protein markers on exosomes. Alternatively, exosomes are molecularly detected using a sandwich assay, where the vesicles are first captured onto the sensor chips using exosome markers such as tetraspanin CD9 and CD63 for exosomes, then detected using targeted surface markers detection Abs. The functionalization of the chip’s surface can cause a slight background response of the sensor surface. When exosomes are introduced into the sensor device, a significant SPR spectra shift is produced by binding the anchored detection Abs to the targeted surface marker on the cancer exosomes. Due to the variance in the expression levels, the binding of different surface markers induces specific degrees in the SPR spectra shifts, allowing for accurate detection of specific biomarkers on exosomes from different origins. This technique also has a fast response time, usually within five minutes of the sample introduction. The SPR methods can detect exosomes in the lower $10^6$/mL concentrations, with the ability to analyze clinical samples for cancer diagnosis also being demonstrated. The sensor response can also be detected by a CCD camera, a method called SPR imaging (SPRi). Liu et al. reported a small and compact intensity-modulated SPR biosensor (25 cm × 10 cm × 25 cm) for exosomal protein detection in the clinical setting. This sensor consisted of a single prism, a slight rotation stage, a continuous wave solid-state laser at 785 nm, and a splitter. They used epidermal growth factor receptor (EGFR) and programmed death-ligand 1 biomarkers. The group used this compact SPR sensor to detect exosomal EGFR derived from non-small cell lung cancer at a concentration of $2 \times 10^{10}$ exosomes/mL.
In 2014, Im et al. engineered a periodic nanohole array device that enhanced the sensitivity and throughput of the SPR technique by developing a landmark nPLEX sensor.\textsuperscript{160} This sensor was used to transmit an SPR spectrum of the periodic nanohole array, offering an improved sensitivity due to electromagnetic enhancement at the nanoholes. The chip consisted of a lattice containing $44 \times 32$ nanoholes on 200 nm thick Au film adhered to a glass substrate, where each nanohole was 200 nm in diameter with a hole-to-hole periodicity of 450 nm. The detection Abs were attached to the Au chip using a polyethylene glycol (PEG) (molecular weight: 2000) linker. Exosomes were injected into the flow cell, and the associated spectral shift of the target-specific proteins on the exosomes was measured. The nPLEX sensor produced sensitivities of $10^3$ exosomes, $10^4$ times higher than the western blot and 100 times higher than ELISA. The captured exosomes were then additionally labeled with spherical AuNPs, improving the signal by 20\%, as well as star-shaped NPs for secondary labeling, producing an enhancement of 300\% over the non-labeled exosomes. This high throughput analysis method was achieved by integrating the nPLEX chip with a multi-channel microfluidic cell where each channel could span over three sensing units. Using an imaging setup, these devices can simultaneously measure SPR transmission intensities of $12 \times 3$ nanohole arrays. Using the nPLEX and ascites samples from ovarian cancer patients, the authors identified exosomal CD24 and EpCAM as specific biomarkers for ovarian cancer diagnostics.

Four years later, the same group developed the nanohole-based SPR assay into a platform that could analyze both intravesicular and transmembrane proteins, as seen in Figure 2.2.\textsuperscript{162} This intravesicular nanoplasmonic system (iNPs) involves the lysing of vesicles to release their intravesicular proteins, binding said proteins to an Ab-functionalized iNPs chip, and further labeling the captured proteins with AuNPs to enhance the SPR shift. The AuNPs enhanced the
Figure 2.2: AuNP-amplified iNPS assay for detecting intravesicular and transmembrane proteins. (A) Schematic of the methodology for protein detection. Exosomes are lysed to release proteins. Targeted proteins are captured on the iNPS chip via affinity ligands and detected by Abs on AuNPs. (B) Scanning electron microscope image showing AuNPs on iNPS sensor. (C) Finite difference time-domain (FDTD) simulation shows the electrical field concentration on AuNPs. (D) Comparison of the signals with and without AuNPs on the iNPS sensor. (E) Protein profiling of exosomes derived from ovarian cells. (F) Drug (HSP90 inhibitor) response monitoring of OV90 cells by exosome protein detection with iNPS. The fold change is the protein level changes monitored by the iNPS spectral shifts after drug treatment. Reprinted with permission from [161]. Copyright {2018} American Chemical Society.

SPR shift response nine times higher than the exosome binding without AuNPs. The iNPs allowed 100 markers (10 × 10 array) to be simultaneously analyzed while achieving a sensitivity of 0.5 μL sample per marker. Im et al. were able to monitor drug responses and found specific drug-dependent exosome protein signatures using their iNPs system. The enhancement of SPR sensitivity using other nanotechnology methods has also been reported.\textsuperscript{231–234} Thakur et al. annealed a Au-coated glass slide using a self-assembly gold nanoislands chip as the SPR sensor, achieving a LOD of 0.194 μg/mL and a linear dynamic range of 0.194-100 μg/mL\textsuperscript{231} This sensor successfully detected and distinguished exosomes, microvesicles, cells from one another in both
urine and serum, using a mouse model. Wang et al. demonstrated an assay to enhance traditional SPR sensor methods using a dual AuNP-assisted signal amplification approach, alternatively using nanostructures on Au film. This SPR chip was used to capture exosomes, which were then bound with aptamer/T30 target surface proteins linked AuNPs, followed by AuNPs coated with A30, binding to the aptamer/T30 linked AuNPs. This assay achieved a LOD of $5 \times 10^3$ exosomes/mL, approximately $10^4$ times more sensitive than ELISA. The AuNP-enhancement mechanism was also achieved for the SPRi sensors. Recently, Fan et al. engineered a microarray SPRi chip using a multiplexed detection method applied with the AuNP-enhanced SPRi assay, achieving a LOD of $10^7$ exosome/mL.

3. Localized Surface Plasmon Resonance Light Scattering Detection

Using AuNPs to detect exosome surface protein markers based on LSPR scattering properties was first demonstrated by Liang et al. in 2017. This nanoplasmonic-enhanced scattering (nPES) assay used Ab-conjugated spherical AuNPs to target one marker such as CD63 and Ab-conjugated AuNRs to target another marker such as CD9. Since the spherical AuNPs scatter light in the visible region and AuNRs scatter in the NIR region, they are detected as green and red particles, respectively, under DF imaging. Allowing for the two targeted surface markers to be detected simultaneously, but when the spherical and rod-shaped AuNPs were close to each other, their LSPR exerted coupling, leading to a third color of yellow, with increased scattering intensity. The appearance of the yellow particles suggests a co-expression of the two surface markers on the same exosome. This method’s sensitivity is very high due to the AuNPs being detected at the single particle level under DF microscopy. Combing CD81 Abs as the capture approach for purification-free exosome and a multi-well glass slide as the sample sensor, this nPES assay is an easy-to-use while consuming low sample samples, 1 µL of plasma. While using the nPES assay, this group
identified ephrin type-A receptor 2 (EphA2) as an exosome marker for diagnosing pancreatic cancer, demonstrating that EphA2-positive exosomes can be used for monitoring tumor progression and treatment response, outperforming the conventional ELISA technique.

4. Surface-Enhanced Raman Scattering Detection

Molecular detection of exosomes via SERS has been reported in two approaches: label-free and labeled assays with SERS nanotags. In label-free detection, exosomes are deposited onto the SERS substrate. Irregular metal surfaces, Au, or Ag nanopatterns are frequently used on 2D substrates. Molecular Raman signals from exosomes such as protein, nucleic acid, and lipid are enhanced by the plasmonic substrates, then detected by a Raman spectrometer. Tirinato et al. fabricated Ag nanograins on silicon (Si) micropillar to enhance the Raman signals from exosomes deposited onto the SERS substrates. It was found that there was a difference in the SERS signals from healthy and tumor cell exosomes. While exosomes from the healthy cells expressed higher intensities of the peaks corresponding to lipid vibrations, exosomes from colon cancer cells exhibited higher intensity signals from the RNA region.

In another study by Park et al., spherical AuNPs were deposited onto a cover glass and dried, as shown in Figure 2.3A. Purified exosomes from H522 and H1299 lung cancer cell lines and human pulmonary alveolar epithelial cells were deposited onto the AuNP-decorated cover glass, and SERS signals from the exosomes were measured. The SERS signals from two different lung cancer cell lines showed a differentiation from one another, as well as the alveolar epithelial cells and the buffered phosphate solution control. Furthermore, using principal component analysis showed clear clusters of the four groups, demonstrating the ability to classify exosomes by SERS detection.
Figure 2.3: SERS-based molecular detection of exosomes. (A) Exosome classification by SERS detection of exosomes from normal and lung cancer cells. Left: Schematic of SERS detection of exosomes. Middle: SERS spectra of exosomes from different origins and control using DPBS. Right: Principal component scatter plot showing clusters of exosomes from different origins. Reprinted with permission from (Ref 238). Copyright (2017) American Chemical Society. (B) Individual molecular detection of exosome-like vesicles by label-free SERS spectroscopy. Left: Schematic of single exosome detection. Each spectrum was recorded from one vesicle to another by moving the laser to a different spatial location of 1, 2, 3, etc. Middle: Representative SERS spectrum of exosomes derived from B16F10 melanoma cells. Right: Representative SERS spectrum of exosomes derived from red blood cells. \(^{242}\) Copyright 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (C) Molecular detection and analysis of exosomes with SERS AuNRs. Left: Schematic of the detection principle showing exosome capture with target-specific Abs on an Au array device and exosome detection with SERS AuNRs via electrostatic interactions with exosome membranes. Middle: Detection of different surface protein markers on exosomes derived from SK-BR-3 breast cancer cells. Right: Detection of HER2-breast cancer by quantification of HER2-positive plasma exosomes. \(^{179}\)
In 2016, Stremersch et al. achieved a label-free SERS detection of individual exosomes, shown in Figure 2.3B.\textsuperscript{242} In this study, exosome-like vesicles were coated with 10 nm AuNPs via electrostatic adsorption. As a result, AuNPs were densely packed, enhancing the SERS signals from the vesicles and allowing for the detection of each vesicle by Raman microscopy. In addition, they could distinguish vesicles from different origins, such as melanoma exosomes and red blood cell exosomes, using partial least squares discriminant analysis on the obtained spectra.

Targeted surface markers on exosomes need to be detected with SERS-active nanotags with Raman reporters.\textsuperscript{243,244} In 2018, Kwizera et al. tested a simple, inexpensive, and portable Raman exosome assay to detect surface protein markers on exosomes attached to SERS AuNRs shown in Figure 2.3C.\textsuperscript{179} In this study, a miniaturized Au array device was fabricated for multiple analyses by assembling a three-dimensional (3D) printed multi-well template and gold-coated microscopy glass slide. Exosomes were purified by ultracentrifugation, then captured onto the apparatus using Abs targeting surface markers of interest. The AuNRs were adsorbed with a QYS21 Raman tag to detect the captured exosomes to create SERS nanotags. The nanotags were then adsorbed onto the exosomes by electrostatic interactions between the positively charged cetyltrimethylammonium bromide on the surface of AuNRs and the negatively charged lipid membrane of exosomes. Using a portable Raman spectrometer, they quantitatively detected the exosomes on the Au device, therefore detecting different surface protein markers using different target capture Abs. They identified HER2 and EpCAM as exosomal biomarkers for detecting HER2-positive BC by analyzing plasma samples from healthy donors and BC patients.
5. Colorimetric Detection

The lateral flow assay (LFA) is a common point of care detection. The principle of LFA is based on transporting liquid samples via capillary force on a 2D polymeric strip that molecularly interacts with the analytes in the fluid samples. The presence of the analytes is detected colorimetrically based on the absorbance properties of the detection labels by the naked eye. LFA is a simple, rapid, cost-effective device suitable for disease screening. A common strategy for LFA using Abs as a detection agent is called lateral flow immunoassay (LFIA). In this method, AuNPs are often used as the detection label. The particles are lined with Abs to recognize antigens for detection. Since the absorption of AuNPs is in the visible region, around 520 nm, they are visible by the eye, making the assay widely accessible to the general public.

In 2016, Oliveira-Rodriguez et al. reported the use of LFIA for the detection of exosomes. In this study, anti-CD9 and anti-CD63 monoclonal Abs were conjugated to 40 nm AuNPs for exosome detection. Purified exosomes must be used, and if they present were in the liquid sample, the exosomes were sandwiched between the AuNP conjugates and anti-CD81, immobilizing the exosomes in the nitrocellulose membrane of the strip, giving a test line. In contrast, the unbound AuNP conjugates migrated further to provide a control line. This method can be completed within 15 minutes and has been tested for the reliability of detection for exosomes in cell culture supernatants, human plasma, and urine, with a LOD of $8.5 \times 10^8$ exosomes/mL. A year later, in 2017, the same research group achieved a multiple-targeted assay, incorporating multiple capture lines. They compared three different nanomaterials: AuNPs, magnetic nanoparticles, and carbon black, for colorimetric readouts and concluded that the AuNPs gave the best results.

Jiang et al. demonstrated another multiplexed facile colorimetric detection platform using AuNPs, functionalized with a panel of aptamers, using color change by aggregation. In the
presence of exosomes, specific aptamers bound to targeted surface proteins on the exosomes cause the aptamers to displace from the AuNPs, inducing aggregation of AuNPs. The AuNPs aggregation of individual particles caused a redshift of LSPR, producing a color change from red to blue.

Di et al. produced an alternative colorimetric method using the enzymatic activity of AuNPs to generate colorimetric detection with 3,3′,5,5′-tetramethylbenzidine (TMB), and hydrogen peroxide (H₂O₂). In this approach, AuNPs were formed on the surface of exosomes by the reduction of Au (III) chloride trihydrate (HAuCl₄) with sodium borate hydride (NaBH₄). In the presence of TMB and H₂O₂, AuNPs attached to the surface of exosomes catalyzed the oxidation of TMB by H₂O₂. This induced a color change in the solution detectable by either the eye or an absorption spectrometer. This method readily distinguished multiple proteins with an ELISA plate reader, differentiating HCC patients from hepatitis B patients and healthy donors. This suggests that this method is a potentially strong candidate for diagnostics.

6. Electrochemical Detection

An electrochemical biosensor is a device for detecting biomarkers by converting molecular interactions into electronic readout signals. It has been widely used as a form for exosome detection. Nanomaterials can be used in electrochemical detection systems to help improve the signal readout. Hu et al. used silver nanoparticles (AgNPs) to enhance the differential pulse voltammetry signals for detecting renal binding proteins. AuNPs have been widely used to enhance the electrochemical detection of DNA.

In 2016, Zhou et al. reported electrochemical detection of the captured exosomes/microsomes with copper (Cu) NPs and AgNPs. In this study, exosomes or microsomes were captured onto an aptamer-modified microfabricated chip containing 11 individual circular Au electrodes. Ab-conjugated AgNPs were used to report EpCAM, while Ab-
conjugated CuNPs were used to account for prostate-specific membrane antigen (PSMA), a biomarker for prostate cancer. The electro-oxidation of these metal NPs induced an electronic response for signal readout, attaining a LOD of 50 exosomes per sensor. Using this sensor, the authors reported that prostate cancer patients showed a significantly higher level of EpCAM and PSMA-positive exosomes, suggesting good potential for prostate cancer diagnostics.

7. Other Detection Methods

Other detection methods involving plasmonic nanomaterials have also been reported.262–272 One method used cantilever arrays to respond to the molecular binding of multiple targets.262 Ab-bound AuNPs were used to detect exosomes captured on the microcantilever, detecting CD24, CD63, and EGFR on exosomes simultaneously and having a LOD of approximately 0.1 pg/mL.

Another method applied AuNPs for amplifying surface acoustic wave (SAW) for detecting and quantifying exosomes reported by Wang et al.268 Exosomes were captured onto the SAW chip, functionalized with anti-CD63 Abs. Streptavidin-conjugated AuNPs recognized exosomes via biotin-conjugated EpCAM that bound to exosomes in advance. As a result, this sensor reached a high sensitivity, with a LOD of $1.1 \times 10^3$ exosomes/mL. In addition, the AuNPs enhanced signals by two orders of magnitude of the SAW chip.

In 2017, Betzer et al. demonstrated a further exciting application of AuNPs for in vivo imaging and tracking of exosomes.263 In their studies, exosomes derived from mesenchymal stem cells (MSCs) were labeled with AuNPs via active uptake of glucose-modified AuNPs. The AuNP-labeled MSC exosomes were administrated in vivo in a mouse model via intranasal or intravenous administrations. Inductively coupled plasma spectrometry was used to quantify the amount of Au at 24 h post-injection, showing a substantial accumulation of exosomes in the brain. In an animal stroke model, computed tomography imaging spotted and tracked in real-time the exosome
migration and accumulation in the stroke area of the mouse brain. They were able to detect as early as a one-hour post-intranasal injection. This exosome labeling and in vivo imaging technique can be a powerful tool for diagnosing various brain disorders.

In addition, AuNPs can work, in tandem, with fluorophores to detect exosomes using the energy transfer process between plasmonic NPs and fluorophores.\textsuperscript{264,265} This energy transfer leads to a quenching effect on the emission of fluorophores when the fluorophores are in proximity to AuNPs. The emission will resume when the fluorophore leaves the metal NPs. AuNPs exhibit fluorescence and thus can serve as fluorescent labels for exosome detection.\textsuperscript{267} AuNPs can also be used as a pure scaffold to carry reporter molecules or targeting ligands for exosome detection by other detection mechanisms.\textsuperscript{266}
CHAPTER 3: DEVELOPMENT AND OPTIMIZATION OF THE DISVT METHODOLOGIES FOR SINGLE EXOSOME PROTEIN PROFILING

1. Introduction

The need for single vesicle technologies (SVT) for liquid biopsies to detect and analyze cancer biomarkers in body fluids such as blood and urine has become a driving factor in developing better cancer diagnosis and monitoring tools. For liquid biopsies to become the gold standard in the clinical setting, they require relatively simple sample preparation, ease of acquisition, low reagent usage, and small sample volumes. The current standardized methods and procedures are considered confusing and over-complicated, but with future technological developments, it is possible. With these issues, there is an increasing need to simplify liquid biopsy methods for early cancer detection using exosomal biomarkers for the clinical setting. The most common liquid biopsy SVT methods are circulating tumor cells, circulating tumor DNA, and exosomes, with exosomes showing the great advantages.

To develop a robust SVT for exosome binding, several critical factors are needed for determining the exosome detection sensitivity and specificity, ranging from the quality of the Au chamber slide to image acquisition. This chapter reports the progress of the development and optimization of DISVT methodologies for single exosome protein profiling. This DISVT used the strong surface plasmon light scattering properties of AuNPs to detect targeted surface protein markers on individual exosomes. Exosomes were directly captured using thiolated CD81 from a diluted cell line stock or plasma samples. Targeted surface proteins were functionalized and conjugated to AuNP, then labeled on exosomes for the protein expression and imaged using DF microscopy for detection. In addition, all exosomes were labeled with a fluorescent nanotag for localization for fluorescent imaging. The combination of both images allows the exosome’s
position, as well as the relative expression of the protein on the exosome surface, for determination. A single exosome’s profiling data from these image sets were generated within minutes using a dual imaging analysis program. The DISVT follows a straightforward capture and labeling procedure, detecting individual exosomes and their surface protein markers on a chamber slide with dual light scattering/fluorescence imaging, which can be fully automated for facile and quick analysis in clinical settings.

2. Fabrication and Characterization of Au Chamber Slide

A thin Au film allows facile surface chemistry through robust thiol chemistry. To ensure high-quality imaging, an ultraflat, uniform, and clean Au surface is needed. Initially, a glass slide (75 mm × 25 mm × 1 mm) sputtered with Au was used, but issues of clustering and irregular binding of the thiolated CD81 kept appearing. From this issue, we further investigated what was causing this problem and discovered that the gold sputtering was uneven and sporadic on the surface. Figure 3.1A is a DF image showing the issues from the sputtered Au surface on the glass slide. It was then concluded that we needed to find a better source for our Au substrate. It was decided that a template stripping method using evaporated Au was ordered from Angstrom Engineering. We found that the issues were resolved using this method, giving a consistent and homogeneous layer for the thiolated CD81. DF imaging showed that the Au surface on the glass slide is clean, flat, and free of defects, as seen in Figure 3.1B.

The template stripping method was achieved by gluing a glass slide onto a Au-coated Si wafer with epoxy at an elevated temperature, then striping the glass slide from the wafer right before use. Because the wafer is the flattest available prime-grade Si, the Au film touching the Si side, which is the side used on the glass slide, is ultraflat and uniform via ultrahigh vacuum deposition. The Si also protects the Au film from air contamination during storage. The Au-coated
glass slide is then separated into multiple wells to form a chamber slide using a polyvinyl tap to analyze multiple samples simultaneously.

In summary, the optimized protocol for the Au chamber slide uses a Au-coated (100 nm in thickness) glass slide prepared by template stripping using a Au-coated Si wafer. First, a four-inch Si wafer (Angstrom Engineering, Inc.) was coated with a thin layer of epoxy solution (Epoxy Technology, Inc.) and then applied with a standard glass slide (8 × 15 mm²), followed by heating for 2 h at 150 °C. After cooling to RT, the glass slide was stripped from the wafer using a razor blade and metal tweezers. Then, a black vinyl tape (~0.2 mm thick) with multiple holes (5 mm in diameter) was applied to the Au surface to form a multi-well Au chamber slide. Each well holds maximally 8 μL of solution. A schematic of the template stripping method can be seen in Figure 3.2.

**Figure 3.1:** Comparison of darkfield images of Au chamber slide made by different methods. (A) Au Sputtered slide. (B) Template stripped Au slide.

**Figure 3.2:** Template stripping. (A) Schematic of the template stripping method to prepare ultraflat, uniform, and clean Au film onto the glass slide.
3. **Functionalization of Au Chamber Slide**

Direct molecular capture has remarkable advantages, including simplified workflow and avoidance of artifacts and aggregates arising from exosome purification steps, ensuring high-purity exosomes, analysis of exosomes with limited volume, practical for multiple samples, and monitoring exosomes in real-time. In addition, using CD81-specific ligands to capture exosomes can dramatically decrease the contamination of normal exosomes, enhancing the sensitivity of detecting tumor-derived exosomes.

CD81, a tetraspanin protein, is shown to be a consistent marker for a surface biomarker for the identification of exosomes, also having a low expression on platelet cells, a significant contributor of normal exosomes in plasma. Thiolated CD81 Abs are used for exosome capture, eliminating the need for long sample purification. CD81 is a protein biomarker used to differentiate exosomes from other EVs. Using CD81 as the capture ligand will dramatically decrease the contamination of platelet cells while increasing the sensitivity of detecting tumor-derived exosomes. Although CD81 is not universally expressed on all exosomes, it is commonly expressed on different cancer exosomes.

In the initial approach towards developing our DISVT method, thiolated CD81 was incubated for 5 h at 25 µg/mL. This technique was derived from Kwizera et al. Problems of capturing few exosomes proved problematic. This concentration was then increased to 50 µg/mL with the same 5 h incubation period, again showing a slightly higher binding but still not enough to analyze. It was later determined that because of the size of the CD81 monolayer chain, more time was needed to untangle itself and stand up. Therefore, the time was changed to an overnight (12 h) incubation period. This optimization can be seen in Figure 3.3.
In preparing the CD81 for binding to the Au chamber slide, 20 μg of CD81 monoclonal rabbit Abs (Biolegend) was washed and redispersed in carbonate/bicarbonate (pH ~9.0) to change the Ab buffer solution. Next, the CD81 was functionalized with a thiol group by reacting the Ab with a 1:100 ratio of 10 mM N-Hydroxysuccinimide (NHS)-PEG-SH 1000 (NanoCS) for 2 h at 37 °C.

Figure 3.3: CD81 Optimization for exosome capture. (A) 25ug/mL for 5 h, (B) 50 ug/mL for 5 h (C) 25 ug/mL for 12 h

Next, a 50 mM tris(hydroxymethyl)aminomethane (Tris) Buffer (pH ~8.0) was added and incubated for 5 minutes at RT to quench the reaction. The quenched solution was then purified by transferring the solution to a 10,000 Dalton (Da) distinct molecular-weight cutoff (MWCO) centrifuge filter (Pall Life Science) and washed by centrifugation three times with Dulbecco's phosphate buffer solution (DPBS) (13,500 RPM, 4 minutes). Finally, the newly thiolated CD81 was transferred to a nonstick vial, stored in DPBS with 0.05% (w/v) Tween 20 (DPBST 0.05%) and 0.05% (w/v) sodium azide and stored at 4 °C. A Bradford assay was run to confirm the exact concentration of the CD81-PEG-SH. Figure 3.4 shows the specificity of thiolated CD81 binding to the Au Chamber slide binding of exosomes using two different microscopes (Nikon LV 150N and Olympus BX41 microscope).
4. Capture of Exosomes on the Au Chamber Slide

For an accurate measurement for the characterization of exosomes, the size of the individual population for our DISVT method must be greater than 1000 exosomes for the sample to evaluate each marker expression accurately. Therefore, we have explored four different approaches to confirm the most efficient and optimal conditions for capturing exosomes with the ability to achieve our goal. We initially believed that multiple additions with longer binding times gave us the best result. However, this produced a poly-layering of exosomes that can cause a steric effect for binding AuNPs, reducing binding efficiency. Thus, we tested our rationale using several parameters of multiple additions during 3 h incubation periods and a single addition for a 2 h and 3 h incubation period (Figure 3.5). As a result, the 2 h single addition of exosomes diluted between 20-1000 times (depending on plasma concentration) in DPBS gave the best outcome.
5. Preparation, Characterization, and Optimization of Target-specific AuNPs

5.1. Preparation

To prepare Abs conjugated to AuNPs, 10 μg of cancer marker-specific monoclonal mouse Abs (Biolegend) were washed and redispersed in carbonate/bicarbonate (pH ~9.0) to change the Ab buffer solution. Next, the specific Ab was functionalized with a thiol group by reacting the Ab with a 1:100 ratio of NHS-PEG-SH 1000 for 2 h at 37 °C. Next, a 50 mM Tris Buffer (pH ~8.0) was added and incubated for five minutes at RT to quench the reaction. The quenched solution was then purified by transferring the solution to a 10,000 Da MWCO centrifuge filter and washed three times by centrifugation with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (13,5000 RPM, 4 minutes). A Bradford assay was run to confirm the exact concentration of the Ab-PEG-SH. To conjugate Abs onto AuNPs, Ab-PEG-SH 1000 was incubated with AuNPs overnight (16 h) at 4 °C. The surface of AuNPs was then saturated with
methoxy polyethylene glycol (mPEG)-SH 5000 (NanoCS) by binding with mPEG-SH 5000 for 1.5 h at RT (molar ratio: mPEG-SH 5000/AuNP = 30,000). The Ab-AuNP conjugates were purified by centrifugation (10,000 RPM, 10 minutes) and washed three times with DPBS 0.05%. The Ab-AuNPs were suspended in DPBS 0.05% and 0.05% (w/v) sodium azide and stored at 4 °C before use (Figure 3.6).

**Figure 3.6:** Preparation and characterizations of target-specific AuNPs. (A) Schematic of the functionalization of Ab with thiol linker. (B) Preparation of target-specific Ab-conjugated AuNPs. (C) TEM image of 60 nm AuNPs. (D) Absorption spectrum of the 60 nm AuNPs. (E) HD size of the 60 nm AuNPs before and after conjugation with anti-HER2 Abs measured by DLS.183

5.2. Determination of the Optimal Size of AuNPs for Exosomal Protein Labeling

Target-specific Ab-conjugated AuNPs detected protein markers of interest on exosomes. AuNPs have a strong LSPR in the visible spectral region.279 Therefore, they exhibit intense light scattering properties, offering a class of fluorescence analogs that can be detected at the single particle level.280,281 This provides an exciting opportunity to detect protein markers on individual exosomes with a simple DF microscope. The AuNPs were 60 nm in diameter and had a LSPR at 525 nm as determined by TEM and absorption spectroscopy, respectively seen in Figure 3.6C&D.
Target-specific Abs were directly linked to AuNPs to ensure a high detection specificity. A lipophilic dye, CLS-PEG-Cy5, labeled and localized exosomes on the chamber slide with fluorescence imaging. The emission of Cy5 (peak maximum at 658 nm) has minimal overlapping with the LSPR band of AuNPs; thus, energy transfer between Cy5 and AuNPs is negligible.

**Figure 3.7: Images of 40 nm, 50 nm, and 60 nm AuNPs.** Images were taken using (A) DF Olympus IX 71 (64x), (B) DF Olympus IX 71 (64x), and (C) DF custom Nikon LV 150N (50x) objective.

Next, we needed to determine what size of AuNP would best be suited for exosome detection. We tested 40 nm, 50 nm, and 60 nm particles. First, we tested our HER2-conjugated AuNPs bound to SK-BR-3 cells using an Olympus IX 71 (**Figure 3.7A**). This shows that all three size particles showed up well but were seen most predominantly at 60 nm.
Next, we tested these particles on CD81 captured exosomes, labeled them, and imaged them using the same microscope (Figure 3.7B). These images showed that our 40 nm AuNPs were not seen, but at 50 and 60 nm, AuNPs had more clarity and were easier to see. Finally, we tested the same three sizes of particles on our SVT microscope, Nikon LV 150N. This provided us with the same results. (Figure 3.7C). Again, we found that the 60 nm AuNPs had the most significant binding and significance in our experiment from these three tests.

5.3. Characterization of the Target-specific AuNPs

The size of the Ab-conjugated AuNPs were measured using DLS and compared with bare AuNPs before conjugation. The results show that the mean HD size of the AuNPs increased from 69 to 78 nm after Ab conjugation (Figure 3.8). This suggests that the thickness of the Ab layer was around 4.5 nm. There were no significant differences in the size change between Abs, such as HER2 and isotype IgG control.

![DLS characterizations of the size of AuNPs before and after protein conjugation.](image)

Figure 3.8: DLS characterizations of the size of AuNPs before and after protein conjugation.

To confirm the success of Ab conjugation and their specificity, we used the HER2 Ab as the model, testing the binding capabilities to HER2-positive SK-BR-3 with a comparison to the control AuNPs linked to isotype IgG. As demonstrated by the FC characterizations, SK-BR-3 cells
have high expression of HER2 (Figure 3.9A&B). Furthermore, DF imaging showed high cellular binding of HER2/AuNPs but not the IgG/AuNPs, indicating the high specificity of Ab-conjugated AuNPs (Figure 3.9C&D). The specificity of Ab-conjugated AuNPs was also examined on exosomes. In contrast to low exosome binding for the IgG/AuNPs, high binding of SK-BR-3 cell-derived exosomes was observed for the HER2/AuNPs (Figure 3.9E&F). This suggests that the Ab-conjugated AuNPs can specifically bind and thus detect target-specific surface protein markers on exosomes.

Figure 3.9: Characterization of target-specific AuNPs. (A&B) FC characterization of HER2 expression on SK-BR-3 cells. (A) PE-conjugated HER2 Ab (B) PE-conjugated IgG Abs. (C) DF images of SK-BR-3 cells labeled with HER2/AuNPs. (D) DF images of SK-BR-3 cells labeled with IgG/AuNPs. (E) DF images of SK-BR-3 cell-derived exosomes labeled with HER2/AuNPs. (F) DF images of SK-BR-3 exosomes labeled with IgG/AuNPs.183

5.4. Theoretical Understanding of AuNP Binding to Exosomes

An experimental measurement for the number of bound AuNPs on a single exosome is extremely difficult. Electron microscopic imaging techniques such as TEM can only detect the AuNPs on exosomes but not measure the total number of bound AuNPs because they are 2D
imaging techniques. Thus, to quantitatively understand the binding of AuNPs to exosomes, we performed Monte Carlo calculations, \( n = 4R_{\text{ves}}^2/R_{\text{hAu}}^2 \), where \( R_{\text{ves}} \) is the radius of the exosome and \( R_{\text{hAu}} \) is the hydrodynamic radius of the Ab-bound AuNP, to roughly estimate the theoretical limit of packing Ab-conjugated AuNPs on exosomes of different sizes from 30 to 200 nm (Figure 3.10A); 40, 50, and 60 nm AuNPs were used for a comparative study. The diameters of the Ab conjugates of these NPs were 60, 70, and 80 nm considering the contribution of the PEG shell and the Ab ligand that usually increased HD by 20 nm. The results showed that for 40 nm AuNPs, the maximal number of the Ab conjugates that can bind to exosomes was 1 for 30 nm exosome and increased to 44.4 for 200 nm exosome, indicating that 40 nm AuNPs can detect exosomes down to 30 nm in diameter. However, for 50 and 60 nm AuNPs (the size used in DISVT), the smallest size of exosomes that can be detected was 40 nm. At this lower limit, 1.3 and 1 AuNPs can bind to the exosome for 50 and 60 nm AuNPs, respectively. For a typical exosome of 100 nm size, the theoretical limits of AuNP binding were 11.1, 8.1, and 6.2 for 40, 50, and 60 nm AuNPs, respectively.

The discrete dipole approximation for scattering and absorption of light by irregular particles version 7 (DDSCAT7)\(^{282} \) was used to simulate the scattering properties of AuNPs bound on exosomes. All DDSCAT calculations were performed by assuming a medium refractive index of 1.33, with diameters of 40, 50, and 60 nm, while using the same configurations of the sphere placements on the exosome. First, we explored the scattering profiles from AuNPs bound to exosomes by considering 1, 2, 3, and up to 6 AuNPs. Then, two different placements of AuNPs were attempted: randomly placing spheres on the exosome surface while disallowing overlapping between the spheres and uniformly placing the spheres on the exosome using an equidistant
placement code. In the case of packing two AuNPs, the distance between the particles is significant; therefore, there is minimal coupling between the two AuNPs.

Figure 3.10: Theoretical examinations of AuNP binding to Exosomes. (A) The theoretical limit of Ab-conjugated AuNPs that can bind to exosomes of different sizes by Monte Carlo calculation. The diameters of AuNPs are 40, 50, and 60 nm. (B&C) Calculated light scattering properties of 40, 50, and 60 nm AuNPs packed on exosomes with DDA. The diameter of the exosome was 100 nm.\(^\text{183}\)

However, with three or more AuNPs, the random placements of the particles on the exosome can lead to a close distance between particles. Therefore, we have also considered placements using equidistance on a sphere, and the scattering spectra calculated for particle numbers greater than three are averaged over both configurations. The scattering spectra of these systems are shown in Figure 3.10B. The higher the number of AuNPs on the exosome, the greater
the scattered intensity. Strong plasmonic coupling occurred for 60 nm AuNPs when packing 3 or more AuNPs randomly. The size had a significant effect on the scattering efficiency of the particle. At a 550 nm wavelength, the scattering efficiency of 60 nm AuNPs was about six times higher than that of 40 nm AuNPs. This explained why 40 nm AuNPs were more challenging to detect than 60 nm AuNPs using a regular DF microscope (Figure 3.1).

![Images of 40 nm (A), 50 nm (B), and 60 nm (C) AuNPs.](image)

**Figure 3.11:** DF images of 40 nm (A), 50 nm (B), and 60 nm (C) AuNPs. Images were taken using an Olympus IX71 microscope with a 40x objective. Scale bar: 20 µm.

We further integrated the scattering spectra with wavelengths from 500 to 800 nm, and the total intensity was plotted against the number of NPs, as shown in Figure 3.10C. The total scattered intensity has a linear dependence on the number of AuNPs despite the size of the particle. The larger AuNPs had a more significant slope of the fitting curve, suggesting a higher degree of change in the scattering efficiency than the small particles concerning the difference in the number of AuNPs on the exosome.

### 6. Fluorescence Labeling of Exosomes

In order to localize exosomes on the Au chamber slide, we label exosomes with a fluorescence dye so as to detect them with fluorescence imaging. Since the LSPR of AuNPs spans from ultraviolet up to 700 nm, we need to choose a dye that excites and emits in the NIR region to avoid energy transfer between AuNPs and the dye. Besides the emission wavelength, brightness is one of the other essential factors to consider since exosomes are small. In addition, the dye needs
to be relatively photostable to detect exosomes accurately. Further, the dye needs to be able to stain exosome lipid membranes. Thus, we designed and customized (Nanocs, Inc) a NIR membrane dye, CLS-PEG 2000-Cy5 (CLS-PEG-Cy5), to label exosomes. The hydrophobic CLS intercalates lipid membranes and thus allows for exosome fluorescence imaging by the linked Cy5. PEG chain with MW of 2000 is used to make the CLS and Cy5 water soluble.\(^{283}\) Cy5 is bright, with a quantum yield of 0.27 in DPBS. They are quite stable and have been widely used for fluorescence labeling in various biological and medical applications. The excitation and emission maxima of Cy5 is 633 nm and 670 nm (Figure 3.12). They have limited spectral overlapping with the LSPR of AuNPs and thus limited energy transfer (cross-talking) between Cy5 and AuNPs.

\[ \text{Figure 3.12: Comparison of the absorption spectrum of AuNPs with the absorption and emission spectra of Chol-PEG-Cy5.} \]

In finding optimal labeling conditions, CLS-PEG-Cy5 was initially incubated at RT for 45 min, according to He et al.\(^{284}\) However, the fluidity of the membrane depicts CLS fusion, which directly correlates with an increased temperature with increased binding, allowing for incubation at 37 °C to immensely increase CLS fusion and solubility of the Cy5 solution and decrease the time for incubation. Figure 3.13 compares CD81 captured exosomes labeled for 15 minutes at ~37 °C.
°C and RT. Under optimal conditions of ~37 °C for 15 min, CLS effortlessly fuses with the semifluid lipid bilayer of exosomes compared to RT.

**Figure 3.13: Optimization of Cy5 Labeling.** (A) Exosomes captured with CD81 labeled with Cy5 dye at 37 °C for 15 min. (B) CD81 with no exosomes labeled with Cy5 dye at 37 °C (C) Exosomes captured with CD81 labeled with Cy5 dye at RT for 15 mins.

To examine whether there is cross-talking between the 60 nm AuNPs and Cy5, we labeled one well of our CD81 captured exosomes with only the AuNPs and a second well with only CLS-

**Figure 3.14: Examination of Cross-Talking between Fluorescence and Darkfield Channels.** (A & C) Fluorescent Channel, (B & D) Concurring DF channel (A & B) Exosomes are only labeled with Cy5 (C & D) Exosomes are only labeled with AuNPs
PEG-Cy5. **Figure 3.14** compares the DF and fluorescent channels with CD81-captured exosomes. The results show that there is no cross-talk between the AuNPs and Cy5.

7. **Design and Construction of The Dual Imaging System**

Initially, images were taken with a 50× objective and the laser-illuminated surface from a vertical (~0°) (**Figure 3.15A**). However, the imaged areas of the wells the camera took were much small. This was due to the image being reflected off of five mirrors before getting to the camera causing spatial resolution issues. We also found that the areas illuminated by the laser had a gaussian effect, with the most intense and brightest in the very center, with the intensity weakening as they reached the laser light’s perimeter (**Figure 3.15B**). With these downfalls, the construct of the dual imaging system was changed to our current construct. Images are taken with a 100× objective, the cameras relocated, placed directly over the illuminated image, and the laser was redirected to have an illumination angle changed to ~45° (**Figure 3.15C**). With the current design, the images obtained, the area of the images are much greater with higher clarity, and the fluorescence intensity is more evenly distributed, allowing for a greater area to be illuminated at one time (**Figure 3.15D**).

Dr. Thang Ba Hoang constructed the optimized system on a customized Nikon LV 150N microscope with a 3D nanometer resolution translation stage (Newport, model 9063). A halogen lamp was used for bright- and DF imaging. Fluorescence imaging was accomplished with a Melles Griot continuous-wave Helium (He) laser (model 05-LPH-925, \( \lambda = 632.8 \text{ nm} \)) in an angled direction (~45°, relative to the sample surface) from the side of a 100× objective and a high numerical aperture (\( N_A = 0.8 \)). The angled illumination allows high-resolution fluorescence imaging on a micrometer scale without sacrificing spatial resolution. The excitation laser (continuous wave He laser, Melles Griot) has a wavelength of 632.8 nm to excite the Cy5 dye.
This setup enables us to uniformly image the sample with high resolution and high sensitivity without needing an expensive and complicated commercial confocal microscope. In addition, it takes fluorescence and DF images of exosomes at the exact location (1 second per image).

![Photographic picture of the dual fluorescence/darkfield imaging system and images obtained.](image)

**Figure 3.15: Photographic picture of the dual fluorescence/darkfield imaging system and images obtained.** (A & C) The major components include the Nikon LV 150N microscope, He laser, angled laser excitation, imaging camera, and ultralong working distance 100x objective. (A) Original configuration. (C) Optimized configuration. (B) and (D) Show images were taken using the constructs from (A) and (C) respectively.\(^{183}\)

8. **Image Analysis: from Image J to SEDIA**

For the image processing of our completed experimentation, Image J was used to process each fluorescent image manually. This process took approximately 4 h to process one individual image set. First, each image was broken down into 16 quadrants. From there, the brightness of the first quadrant was adjusted to allow for each particle to be seen. Next, a bandpass filter was run on the quadrant to reduce any artifacts in the image. Next, the threshold was adjusted to make a binary image (black-and-white) and to remove any stray pixels. Next, an outline of each particle was
created from the binary image so it could be used later. This was then repeated for the following 15 quadrants. After all, quadrants were processed, the outlined particles were overlayed to the DF image, and the intensity for each was taken. The final processed image can be seen in Figure 3.16A.

Next, our image processing was then upgraded by Dr. Hoang using LabVIEW Software. LabVIEW software version 4 reduced the image processing to 1-2 h to process one image set (Figure 3.16B). First, each image set was uploaded into the program and the brightness for each image was adjusted to see all particles. Next, an area of the image was selected, and an overlay of the two images was shown in high contrast of a blue background and orange for the particles. The particles were then individually selected and measured for intensity. The problem with this program version was that when brighter particles were adjacent to smaller particles, the smaller particles were hard to process. Furthermore, the more particles an image had, the smaller the selected area to be analyzed, causing processing time to increase.

Next, the LabVIEW software was upgraded to version 7 (Figure 3.16C). This version reduced image processing to 15-45 minutes for each image set. First, each image set was uploaded to the program, an area was selected, and the brightness was adjusted. After the location chosen was optimized, intensities for all particles seen in the designated area were automatically processed. Unfortunately, this version of the LabVIEW software had similar problems as the previous version: brighter particles cause smaller particles not to be seen, and the more particles in an image, the small the selected area could be processed.
Figure 3.16: Development of Image Analysis (A) Image J (B) LabVIEW Version 4 (C) LabVIEW Version 7.
Finally, our SEDIA program was developed, bringing the processing time to approximately 5 minutes to process one image set. Dr. Caleb Galops and Mitch Taylor developed an image analysis platform using Bash scripts, Python, and ImageJ (Figure 3.17). We named the platform SEDIA, which stands for Singe Exosome Dual Imaging Analysis. First, Bash scripts are used to automatically import a set of raw fluorescence images (exosome mask) and DF images (protein target) into ImageJ. Then, Python scripts are used within ImageJ itself using the native Jython interpreter to implement a custom image analysis process utilizing ImageJ algorithms automatically. Next, each set of images is processed with a 2D implementation of a zeroth-order Savitzky–Golay smoothing filter to suppress pixel-to-pixel noise, followed by a $5 \times 5$ custom convolution kernel to reduce unequal illumination. Next, Otsu’s method sets a threshold for detecting spots for each image, and then each image is converted to the binary format. A custom

**Figure 3.17: Image pre-processing with SEDIA using a fluorescent image of exosomes as an example.** (A) Original Image (B) Pixel-to-pixel noise is removed with a Savitzsky-Golay filter (C) Illuminated image produced with convolution by separability (D) Gaussian blur function applied to blur local pixel values (E) Fast-Fourier transform bandpass filter used to restrict ranges of pixel values (F) Otsu threshold used to minimize intra-class variance.
Python script utilizing sci-kit-image is then used to label the Cy5-bound exosomes in the mask image and AuNP-bound exosomes in the target image, followed by overlapping the two images with overlapping label areas of 50% or greater being accepted as marker-positive exosomes. The logarithm of the normalized integrated pixel intensity in the unit area of labeled particles in the DF image at the exosome mask locations gives the population density histogram that includes two populations, AuNP-bound exosomes, and AuNP-free exosomes. To compensate for the variation of instrument responses, the density profile is normalized by defining the maximum intensity of the AuNP-free exosome band to be one (and thus its logarithm to be zero). The fraction of marker-positive exosomes to total exosomes, $F_p$, is then calculated by taking the difference between the fraction of Au-bound exosomes using the Ab-conjugated AuNPs and the fraction of Au-bound exosomes using the IgG-conjugated AuNPs. Finally, the difference in the average normalized intensity of all exosomes between Ab-conjugated AuNPs and IgG-conjugated AuNPs is calculated as $\zeta_p$ to represent the expression level of the targeted protein on individual exosomes.

The protein expression profiles on single exosomes were derived using SEDIA, a semiautomatic imaging analysis platform developed in-house using Bash scripts, Python, and ImageJ. This code analyzes multiple images simultaneously within seconds, which is ~50 times faster than using ImageJ alone, which analyzes one image set at a time (5 minutes with SEDIA vs. 4 h with ImageJ per sample). **Figures 3.18** show the crucial steps of SEDIA using a plasma sample from a HER2-positive stage III BC patient. SEDIA measures the raw pixel intensity of the fluorescence image (exosome mask) and the DF image (protein target).
Figure 3.18: Illustration of labeling, overlapping, and signal extraction during image analysis with SEDIA. Plasma exosomes from a HER2-positive stage III breast cancer patient were used as examples. (A) Plasma exosomes labeled with HER2/AuNPs. (B) Plasma exosomes labeled with IgG/AuNPs. In the overlapped image, AuNP-bound exosomes were shown as overlapped red and orange labels, and AuNP-free exosomes were shown as red labels only. (C&D) Population density histograms showing AuNP-bound exosomes (blue peak) and AuNP-free exosomes (orange peak) after the exosomes were labeled with HER2/AuNPs (C) and IgG/AuNPs (D).

9. Conclusion

In conclusion, we have developed and optimized all the parameters that determine the sensitivity, specificity, and robustness of the DISVT. Figure 3.19 shows the finalized DISVT capture and labeling protocol. Briefly, the template-stripped Au chamber slide was functionalized
with the rabbit CD81 monoclonal Ab (catalog number 349502) thiolated PEG linker (catalog number PG2-NSTH-5K) by incubation. The optimized binding protocol used CD81-PEG-SH 1000 25 μg/mL in 5% bovine serum albumin (BSA) (catalog number 37525) for 12 h at RT. After incubation, samples were washed with DPBS containing 0.01% Tween 20 (Catalog number P9416-100mL) (DPBST 0.01%) three times and then rinsed with DPBS three times. The chamber slide was then saturated with hydrophilic but uncharged 0.1 mM 11-mercapto undecyl tetra (ethylene glycol) (catalog number 674508) (MUTEG) to block nonspecific interactions of the slide with biochemicals in subsequent steps and incubated for 1 h at RT. After incubation, samples were washed with DPBST 0.01% three times and then rinsed with DPBS three times.

Next, exosomes were captured (immobilized) on the Au slide by incubating low-concentration exosomes (∼10^8 exosomes/mL) for 2 h at RT. The low concentration of exosomes is essential to allow detection. It also avoids the steric effect for binding AuNPs to ensure the

**Figure 3.19: Exosome capture, labeling, and characterization.** A schematic of exosome capture and labeling.
detection of small exosomes. After incubation, samples were washed three times with DPBST 0.01% and rinsed with DPBS three times. Then, exosomes were labeled with the target-specific Ab-conjugated AuNPs by incubating the conjugates (50 pM in DPBST 0.05%) for 1 h at RT (flipping every 15 minutes to keep AuNPs from settling on the surface). After incubation, each well was washed three times with DPBST 0.05% and rinsed three with DPBS. Finally, exosomes were labeled with a Cy5 fluorophore for localization of all exosomes by incubating CLS-PEG-Cy5 2000 at 20 μM at 37 °C for 15 min, and each well was then rinsed with DPBS and water three times to remove any excess DPBS crystals and dried with nitrogen.

Then the labeled exosomes are examined with fluorescence and DF images, taken using the highly sensitivity dual imaging system built by Dr. Hoang. Images are then analyzed using the SEDIA method based on Bash script, Python. Data were extracted with SEDIA to give the exosome population density histogram that shows the AuNP-bound and AuNP-free exosomes (Figure 3.20). The fraction of exosomes from all captured exosomes that are positive for the targeted protein marker \( F_p \) will be calculated as the difference of \( F_p \) between the targeted protein p and isotype IgG.

Compared to the SVT reported in the literature, our DISVT has significant advantages in terms of sample consumption, simplicity, speed, and cost. For example, our DISVT only needs less than 10 μL of 10–1000 diluted plasma per assay, based on a straightforward capture and labeling procedure of ~4 h combined with fast dual image acquisitions and analysis (within seconds). In addition, the single particle detection sensitivity of surface plasmon light scattering AuNPs allowed the detection of surface antigens on individual exosomes with a routine optical microscope.
Figure 3.20: Summary of the developed DISVT methodology for single exosome surface protein profiling. (A) Schematic of the principle of exosome capture and surface protein detection. (B) Schematic of the excitation and signal collection of the dual DF/fluorescence microscopic system. (C) Fluorescence image of plasma exosomes from a HER2-positive stage III breast cancer patient. (D) DF image of plasmon exosomes from the patient in (C) labeled with HER2/AuNPs. (E) Overlapping (C) and (D) show AuNP-bound exosomes (overlapped yellow and red labels) and AuNP-free exosomes (red labels only). (F) Population density histogram showing the distribution of AuNP-bound exosomes (blue peak) and AuNP-free exosomes (orange peak).
CHAPTER 4: SURFACE PROTEIN PROFILING OF CELL-DERIVED CANCER EXOSOMES WITH DISVT

1. Introduction

Cancer is a heterogeneous disease produced by the mutation of a group of cells in the human body, resulting in a steady and uncontrolled growth within their original tissues that can damage surrounding organs. BC is the most prominently diagnosed type of cancer and the second cause of death to lung cancer in women worldwide. Two primary factors in tumor growth and proliferation are genetic/epigenetic mutations in the cancer cell and the reorganization of the components of the tumor microenvironment, primarily influencing tumorigenesis and advancement. The tumor microenvironment components consist of but are not limited to normal cells, tumor cells, stromal cells, fibroblasts, endothelial cells, CD8+ cells, macrophages, microglia, blood vessels, lymphocytes, cytokines, exosomes, and extracellular matrix. The main mechanisms contributing to proliferation are cellular and extracellular.

Due to their unique attributes, exosomes and microvesicles hold great potential for sensitive cancer diagnostics. Exosomes are membrane-bound vesicles continuously released into the extracellular environment. Exosomes are generated via exocytosis after the fusion of multivesicular bodies with the plasma membrane and microvesicles by outward budding of the plasma membrane. They carry the DNA, RNA, and various proteins of the parental cells. Exosomes from tumor cells can transfer oncogenic factors through intercellular communication and regulate angiogenesis, immunity, and metastasis to promote tumorigenesis and progression. Tumor-derived exosomes enter the blood (typically $10^9$–$10^{11}$/mL) and many other body fluids such as urine, saliva, ascites, and cerebrospinal fluid.
well as having the ability to be preserved for years when stored in liquid nitrogen. Thus, exosomes offer a rich new source of biomarkers for noninvasive liquid biology in medicine.

This chapter will characterize the surface proteins on cell-derived cancer exosomes using DISVT for single exosome protein profiling. For detection, AuNPs were conjugated with a target-specific protein to label positive exosomes, and all exosomes were also labeled with a fluorescent nanotag captured with CD81. This method was tested with multiple surface protein markers (CD44, HER2, and EpCAM) on exosomes from three BC models (SK-BR-3, MDA-MB-231, and MCF-7). Using the method, we demonstrated that the BC cell lines can be differentiated using target-specific exosome proteins. In addition, AuNP-labeled exosomes were imaged with DF microscopy for detection, and exosomes labeled with the fluorescent nanotag were imaged using fluorescence microscopy for the localization of all exosomes captured. These image sets showed the position of individual exosomes and the relative expression of proteins on the exosomal surface, which could later be profiled using our dual imaging analysis software.

2. Materials and Methods

2.1. Materials

All reagents were purchased from MilliporeSigma (St. Louis, MO) unless otherwise specified. Antibodies were purchased from Biolegend (San Diego, CA). Phycoerythrin (PE) labeled Abs were purchased from Miltenyi Biotec (Auburn, CA). All cell lines were purchased from ATCC (Manassas, VA). Cell culture media was purchased from VWR (Radnor, PA), and fetal bovine serum (FBS) was purchased from Fisher Scientific (Waltham, MA). NHS-PEG-SH (MW 1000), mPEG-SH (MW 5000), and CLS-PEG-Cy5 (MW 2000) were purchased from Nanocs (Boston, MA).
2.2. **Cell Culture and Flow Cytometry Analysis**

Cells were cultured in their respective media with 10\% FBS and 1\% Penicillin-Streptomycin (100×) at 37 °C under 5\% CO\(_2\). The medium was Roswell Park Memorial Institute Medium (RPMI) 1640 for SK-BR-3 and Dulbecco’s modified Eagle’s medium (DMEM) with high glucose for MDA-MB-231 and MCF-7. For the FC analysis of surface protein markers, 10\(^6\) cells were incubated with 2 μg/mL PE-conjugated Abs for 30 minutes at 4 °C. The cells were then washed by centrifugation (15 minutes, 1500 rpm) to eliminate free abs. Finally, the cells were analyzed for the targeted surface markers with a Millipore Guava easyCyte 8HT flow cytometry system.

2.3. **Collection of Cell-derived Exosomes**

The cells were grown in conditioned cell culture media (media + 10\% EV-free FBS) to collect exosomes for 48 h. The culture supernatant was collected and centrifuged at 430g at RT for 10 minutes. The supernatant was collected and centrifuged at 16,500g at 4 °C for 30 minutes. Then, the supernatant was collected and centrifuged at 100,000g at 4 °C for 70 minutes. After removing the supernatant, the exosome pellet was resuspended in cold, sterile DPBS and centrifuged again at 100,000g at 4 °C for 70 minutes. Finally, the exosome pellet was resuspended in cold, sterile DPBS, filtered with a 0.2 μm Polyethersulfone (PES) filter, and stored at −80 °C until analysis.

2.4. **Size characterizations with NTA**

Exosomes were characterized with NTA following the procedures described in our previous studies.\(^{290}\) Briefly, cell-derived exosomes were characterized with NTA using a Nanosight LM10 microscope (Malvern Instruments, Inc., Westborough, MA) to determine the concentration and size. The samples were diluted to keep exosome concentration within the range
of $10^6$–$10^9$ exosomes/milliliter in accordance with the manufacturer’s recommendations. All samples were analyzed in triplicate of 40 seconds videos with the camera level set at 12 and the detection threshold set at 10.

2.5. **ELISA Characterization**

In total, 50 μL of 2 μL/mL rabbit CD81 monoclonal Abs were added to a 96-well polystyrene plate in triplicate and incubated overnight at 4 °C. After three times washing with DPBST 0.05%, 50 μL of 1% BSA was added and incubated for 2 h at RT to block the unbound surface. After washing with DPBS, 50 μL of cell-derived exosomes (1.0 × $10^9$ exosomes/mL) or plasma exosomes (10× diluted with DPBS) were added and incubated at 4 °C overnight. After washing three times with DPBS, exosomes were treated sequentially with the following solutions: 50 μL of 2 μg/mL target-specific Abs (2 h, RT), 50 μL of the anti-mouse secondary Ab conjugated with horseradish peroxidase (HRP, 1:3400 dilution in 1% BSA, 2 h, RT), and 100 μL of 3,30,5,50-tetramethylbenzidine (TMB, 30 min, RT), with three times washing using DPBS between steps. Oxidation of TMB was stopped with 100 μL of 2 M sulfuric acid (H₂SO₄). The optical density was measured at 450 nm using a BioTEK ELx800 microplate reader. Isotype IgG was replaced as the negative control for the primary Ab step.

2.6. **Sample Preparation and Data Collection with DISVT**

Samples were prepared following the developed methodology described in Chapter 3. Briefly, a 4-inch Si wafer was coated with a thin layer of epoxy solution and then applied with a standard glass slide, followed by heating for 2 h at 150 °C. After cooling to RT, the glass slide was stripped from the wafer using a razor blade and metal tweezers, which led to a uniform and ultraflat Au film on the glass slide. Then, a section of black polyvinyl tape with multiple 5mm holes was applied to the Au surface to form a multi-well Au chamber slide. The Au chamber slide was then
functionalized with the rabbit CD81 monoclonal Ab thiolated PEG linker by incubation. The optimized binding protocol used CD81-PEG-SH 1000 25 mg/mL in 5% BSA for 12 h at RT. After incubation, samples were washed with DPBS containing DPBST 0.01% three times and then rinsed with DPBS three times. The chamber slide was then saturated with hydrophilic but uncharged 0.1 mM MUTEG to block nonspecific interactions of the slide with biochemicals in subsequent steps and incubated for 1 h at RT. After incubation, samples were washed with DPBST 0.01% three times and then rinsed with DPBS three times.

Next, exosomes were captured (immobilized) on the Au slide by incubating low-concentration exosomes (\(\sim 10^8\) exosomes/mL) for 2 h at RT. The low concentration of exosomes is essential to allow detection. It also avoids the steric effect for binding AuNPs to ensure the detection of small exosomes. After incubation, samples were washed three times with DPBST 0.01% and rinsed with DPBS three times. Next, exosomes were labeled with the target-specific Ab-conjugated AuNPs by incubating the conjugates (50 pM in DPBST 0.05%) for 1 h at RT (flipping every 15 min to keep AuNPs from settling on the surface). After incubation, each well was washed three times with DPBST 0.05% and rinsed three with DPBS. Finally, exosomes were labeled with a Cy5 fluorophore for localization of all exosomes by incubating CLS-PEG-Cy5 2000 at 20 \(\mu\)M at 37 °C for 15 min, and each well was then rinsed with DPBS and water three times to remove any excess DPBS crystals and dried with nitrogen. DF/fluorescent images were then acquired using the dual imaging system and then analyzed using the SEDIA program.

2.7. Statistical Analysis

Statistical analysis was performed to compare the expression levels of targeted proteins on human subjects of different groups using analysis of variance (ANOVA) with the post hoc Scheffe method. A \(p\)-value \(\leq 0.05\) was considered significantly different. The mean difference between
different groups was considered significant if the absolute value was greater than the considerable minimum difference derived from the Scheffe method. In addition, the diagnostic value of HER2 in BC patients was evaluated by receiver operation characteristic (ROC) curve analysis using R packages.

3. Results and Discussion

3.1. Characterization of Surface Protein Expression on BC cells with Flow Cytometry

To validate the specificity and sensitivity of DISVT, we selected three common BC cell lines, SK-BR-3, MDA-MB-231, and MCF-7, as our cell models. SK-BR-3 is a HER2-positive BC cell line, showing an increased expression of EpCAM and a low expression of CD44. MDA-MB-231 is a triple-negative metastatic BC cell line (HER2, ER, and PR negative). It is shown to have a high expression of CD44 with low expressions of HER2 and EpCAM. MCF-7 has a high expression of EpCAM with a moderate expression of CD44 and a low expression of HER2. MCF-7 cells have a high expression of EpCAM, a moderate expression of CD44, and a low expression of HER2. This knowledge is well known and reported in many literatures.

To confirm this knowledge, we used flow cytometry to characterize the expression profiles of EpCAM, HER2, and CD44 on three BC cell lines. In agreement with general knowledge, SK-
BR-3 cells are EpCAM and HER2-positive, MDA-MB-231 cells are CD44-positive, and MCF-7 is EpCAM- and CD44-positive with moderate expression of HER2 (Figure 4.1).

3.2. NTA Characterization of The Size of Exosomes

NTA was used to determine the HD sizes for exosomes from each cell line. The HD size of exosomes from SK-BR-3, MDA-MB-231, and MCF-7 was 167 ± 38, 168 ± 49, 145 ± 45, and 156 ± 32 nm, respectively (Figure 4.2). Each cell line was characterized in a previous study, with analysis showing no statistical difference between exosome sizes between cell lines.

![Figure 4.2: NTA Characterization of cancer cell-derived Exosomes. (A) SK-BR-3, (B) MDA-MB-231, and (C) MCF-7.](image)

3.3. Characterization of Surface Protein Expression of Exosomes with ELISA

We used sandwich ELISA to characterize surface protein expressions on exosomes at the bulk level. This method was used to compare the expression for two common BC-associated surface protein markers, EpCAM, HER2, and CD44, on three BC cell lines, MDA-MB-231 (triple-negative metastatic BC), and SK-BR-3 (HER2-positive BC) (Figure 4.3). The expression profiles of these markers for each cell line were confirmed using ELISA (Figure 4.4). MDA-MB-231 was shown to have a high expression of CD44 with low expressions of HER2 and EpCAM. SK-BR-3 was shown to have a high expression of HER2 with an increased expression of EpCAM and a low expression of CD44.
**Figure 4.3:** Schematic of the sandwich ELISA principle for detecting target-specific surface protein markers on exosomes.\(^{183}\)

**Figure 4.4:** Characterization of surface protein expression using ELISA. EpCAM, HER2, and CD44 characterization on exosome derived from cell lines SK-BR-3 and MDA-MB-231.

3.4. *Surface Protein Profiling of BC Cell-derived Exosomes with DISVT*

Using DISVT, we examined the ability of DISVT to specifically detect and differentiate different surface proteins on exosomes from different origins by profiling EpCAM, HER2, and CD44 on SK-BR-3, MDA-MB-231, and MCF-7 cells. All data were background corrected using...
the signals from Ab IgG-conjugated AuNPs. Representative fluorescence, DF, and overlay images of the exosomes labeled with the three different markers for the three cell lines are shown in Figures 4.5-4.7. The results showed apparent differences in the population of the AuNP-bound exosomes depending on the protein marker and the cell line. The SK-BR-3 exosomes had high AuNP binding for EpCAM and HER2 compared to the IgG control, while the CD44 profile was similar to that of the IgG control. The MDA-MB-231 exosomes had high AuNP binding for CD44 and low binding for HER2 and EpCAM. The MCF-7 cells had high AuNP binding for EpCAM, moderate binding for CD44, and low binding for HER2. These results were consistent with previous studies with bulk methods using SERS or fluorescence spectroscopy.\textsuperscript{179,290}

The results also suggest that exosomes reflect their parental cells in terms of molecular expression, a consensus that this field has reached based on numerous studies. We were analyzing the CD81-positive exosome subtypes rather than the total exosomes as characterized by Lee et al. in their glioblastoma mode.\textsuperscript{156} These studies using model BC cell lines suggest the high specificity of our DISVT-based assay.
Figure 4.5: Exosomes derived from MDA-MB-231 cells. Detection of EpCAM, HER2, and CD44 in comparison to their IgG controls. Scale bar: 5 µm.
Figure 4.6: Exosomes derived from SK-BR-3 cells. Detection of EpCAM, HER2, and CD44 in comparison to their IgG controls. Scale bar: 5 µm.183
Figure 4.7: Exosomes derived from MCF-7 cells. Detection of EpCAM, HER2, and CD44 in comparison to their IgG controls. Scale bar: 5 µm.\textsuperscript{183}
Using SEDIA, we derived exosome density histograms for all three markers on the three cell lines, shown in Figure 4.8. We used CD81 positive exosomes derived from TNBC MDA-MB-231 for our first model, examining the specificity of the target-specific conjugated AuNPs in our assay. Exosomes were labeled with Ab-conjugated AuNPs targeting the three proteins (red) and the IgG-conjugated AuNPs (gray). Figure 4.8A1-3 shows the population density profiles of the MDA-MB-231-derived exosomes after being labeled with the Ab-conjugated AuNPs. The highest expression marker was CD44, ~74%, compared to the negative markers HER2 ~5.0% and EpCAM ~4.2%. The results show that a strong binding was observed from AuNPs for exosomes targeting CD44. In contrast, signals from the two negative controls had little to no representative binding.

Next, we used CD81-positive exosomes derived from HER2-positive BC SK-BR-3 as our second model, examining the specificity of the same target-specific conjugated AuNPs in our assay. Exosomes were labeled with the same Ab-conjugated AuNPs targeting the three proteins (red) and the IgG-conjugated AuNPs (gray). Figure 4.8B1-3 shows the population density profiles of the SK-BR-3-derived exosomes after being labeled with the Ab-conjugated AuNPs. The highest expression markers were HER2 with ~78% and EpCAM with ~70%, compared to the negative marker of CD44 with an expression of ~5.0%. The results show that a strong binding was observed from AuNPs for exosomes targeting HER2 and EpCAM, whereas signals from the CD44 had very little to no binding.

Finally, we used CD81 positive exosomes derived from ER and PR positive, HER2-negative BC MCF-7 as our third model. We examined the specificity of our target-specific conjugated AuNPs in our assay. Exosomes were labeled with Ab-conjugated AuNPs targeting the three proteins (red) and the IgG-conjugated AuNPs (gray). Figure 4.8C1-3 shows the population
density profiles of the cell-derived exosomes after being labeled with the Ab-conjugated AuNPs. The highest expression marker was EpCAM with ~75%, while CD44, a moderately expressed protein, was ~32% compared to the negative marker of HER2 with an expression of ~2.3%. The results show that a strong binding was observed from AuNPs for exosomes targeting EpCAM, whereas signals from the CD44 showed less binding, and HER2 showed little to no binding.

Figure 4.8: Single exosome surface protein profiling of cancer cell-derived exosomes with DISVT. Population density histograms of the surface expression of (A) MDA-MB-231, (A1) EpCAM, (A2) HER2, and (A3) CD44. (B) SK-BR-3 (B1) EpCAM, (B2) HER2, and (B3) CD44. (C) MCF-7, (C1) EpCAM, (C2) HER2, and (C3) CD44, labeled with target-specific Ab-conjugated AuNPs (red) and IgG-conjugated AuNPs (grey).
3.5. Correlation of ELISA and DISVT

To validate the accuracy of DISVT for surface protein characterization, we determined $\xi_p$ for each marker on the exosomes from each cell line. Then, we compared it with the sandwich ELISA. ELISA has been used to validate SVT since a golden standard for SVT does not exist yet. As mentioned above, $\xi_p$ is the average normalized pixel intensity of the DF images on all analyzed exosomes; thus, it is comparable to bulk analysis. Furthermore, both DISVT and sandwich ELISA used CD81 to capture exosomes. Therefore, our studies can use sandwich ELISA to validate DISVT with $\xi_p$. The results showed that the two methods were highly correlated, with a Pearson correlation coefficient of 0.98 (Figure 4.9). This correlation demonstrated that DISVT could reliably detect different surface protein markers on exosomes from different origins. Furthermore, it shows that exosome surface protein profiles reflect those of their parental cells.

![Figure 4.9: Correlation of DISVT-$\xi_p$ and ELISA for surface protein marker detection on cell-derived exosomes.](image)

$y = 1.3x - 0.15$

$R^2 = 0.98$
4. Conclusion

In conclusion, we have demonstrated that surface markers on BC cell lines derived from exosomes can be profiled and quantified using our DISVT system. In this method, we showed that DISVT was able to profile and quantify exosome subtypes specific to targeted BC markers (EpCAM, HER2, and CD44) for three BC cells (SK-BR-3, MDA-MB-231, and MCF-7). In addition, we have shown the ability to detect surface protein markers on exosomes, differentiating cancerous from normal exosomes using cancer-associated surface protein markers. The expression profiles of the biomarkers for each cell line were confirmed using flow cytometry. Finally, we have also validated our DISVT using a sandwich ELISA, demonstrating that DISVT could detect different surface protein markers on exosomes from different origins.

Our technology is sensitive, simple, and efficient, with low consumption of samples. With proof-of-concept studies, we demonstrated our DISVT on three exosome populations derived from our BC cell lines SK-BR-3, MDA-MB-231, and MCF-7. Further, we have validated our results with widely used flow cytometry methods and sandwich ELISA. In addition, our SVT method can be adjusted for any protein marker or cancer cell line without changing the methodology. The next step in the validation process of our method is to test exosomes plasma derived from various stages of HER2-positive cancer as well as the healthy donor.
CHAPTER 5: APPLY DISVT TO EVALUATE THE POTENTIAL OF PLASMA EXOSOMES FOR EARLY CANCER DETECTION USING HER2-POSITIVE BREAST CANCER AS THE DISEASE MODEL

1. Introduction

Exosomes are membrane-bound vesicles of cellular origin continuously released via exocytosis into the extracellular matrix. They can be found in almost all types of body fluids such as blood, saliva, urine, and breast milk. Exosomes are composed of lipids, proteins, nucleic acid, metabolites, coding and noncoding and RNA. Exosomes can reflect the pathological state of the donor cells making giving them great potential as markers for monitoring and treatment. HER2 is a known cancer protein marker to be overexpressed in various primary cancers. It has been reported that HER2 is overexpressed in several types of cancer, particularly BC. These changes can control many cellular pathways, such as proliferation, angiogenesis, and metastasis, enabling oncogenesis and cancer development.

Early diagnosis is a crucial period for reducing cancer deaths. Using exosomes as biomarkers is a relatively new field, allowing for detection, characterization, and treatment response in cancer patients. The significance of the exosome’s composition and functions has driven novel developments of various investigative methods for detecting proteins, lipids, and other genetic material, as stated in previous chapters. However, molecular detection is exceptionally challenging due to the exosome's small size: low amounts of antigens, a low refractive index, and interference caused by aggregates in the sample.

Surface proteins are the contact point for cell-to-cell communication. Oncogenic receptors often reside within regions of the plasma membrane. Thus, tumor-derived exosomes may be probed by detecting surface oncogenic proteins on exosomes in body fluids. Many studies have
used bulk methods. The traditional ELISA, Western Blotting, requires a minimum of $10^5$-$10^6$ exosomes per biomarker\(^{156}\) to measure a single biomarker or emerging techniques based on various detection mechanisms to detect surface proteins on exosomes.\(^{177,179,206,230,231,276,305,306}\) However, exosomes have a heterogeneous composition and masking tumor-derived exosomes mixed with a vast background of non-tumor exosomes from various tissues and hematopoietic cells. Furthermore, the exosome concentrations in body fluids are highly dependent on the number of cells producing them,\(^{307}\) concluding early stages cancers produce fewer exosomes from cancer cells.\(^{296}\) Finally, it has been reported that blood has one of the most abundant body fluids supplies of exosomes, with concentrations estimated between 5-15 $\times$ $10^8$ particles/mL.\(^{307}\) The ability to sensitively, precisely, and quantitatively analyze target-specific exosome subtypes for basic vesicle research and clinical applications is highly desirable to analyze exosomes at the single vesicle level.

In this chapter, we will report on a facile DISVT to evaluate the potential of exosomes for early cancer detection using HER2-positive BC as the disease model for single exosome protein profiling is reported. Using HER2-positive BC as the disease model, we will demonstrate that this DISVT can quantify the fraction of tumor-associated exosomes in plasma samples and detect BC at early stages. In contrast, the traditional bulk ELISA can only detect BC at a locally advanced stage. The DISVT also precisely differentiated HER2-positive BC from HER2-negative BC. We will also show that the amount of tumor-associated exosomes tripled in locally advanced patients compared to that in early-stage patients. Our method is simple and rapid with low sample consumption, which makes it highly promising for cancer screening and real-time monitoring.
2. Materials and Methods

2.1. Materials

All reagents were purchased from MilliporeSigma (St. Louis, MO) unless otherwise specified. Abs were purchased from Biolegend (San Diego, CA). Phycoerythrin (PE) labeled Abs were purchased from Miltenyi Biotec (Auburn, CA). All cell lines were purchased from ATCC (Manassas, VA). Cell culture media were purchased from VWR (Radnor, PA), and FBS was purchased from Fisher Scientific (Waltham, MA). NHS-PEG-SH (MW 1000), mPEG-SH (MW 5000), and CLS-PEG-Cy5 (MW 2000) were purchased from Nanocs (Boston, MA).

2.2. Source of Exosomes from Patients and Healthy Donors

Human plasma from healthy donors and BC patients was obtained from BioIVT, Inc. The samples were not collected explicitly for our studies, and we could not access the subjects’ identifying information. Based on U.S. Department of Health & Human Services regulations, our research is non-human subject research. The plasma was diluted with DPBS 20–1000 times depending on the subjects (final concentration of ~10^8 exosomes/mL) and filtered with a 0.2 μm PES filter (Agilent Technologies) before use.

2.3. Characterization of Exosomes with NTA and SEM

Exosomes were characterized with NTA and SEM following the procedures described in our previous studies. Briefly, plasma or cell-derived exosomes were characterized with NTA using a Nanosight LM10 microscope (Malvern Instruments, Inc., Westborough, MA) to determine the concentration and size. The samples were diluted to keep exosome concentration within the range of 10^6–10^9 exosomes/mL in accordance with the manufacturer’s recommendations. All samples were analyzed in triplicate of 40-second videos with the camera level set at 12 and the detection threshold set at 10. For SEM imaging, plasma exosomes from a HER2-positive BC
patient were fixed with 2% glutaraldehyde, purified by ultracentrifugation, and then placed on a Si chip. After drying, exosomes were coated with a 2–3 nm Au film and then imaged with a Nova NanoSEM650 field emission SEM. Images were taken with a voltage of 15 kV and a magnification of 20,000×.

2.4. ELISA Characterization

In total, 50 μL of 2 μL/mL rabbit CD81 monoclonal Abs were added to a 96-well polystyrene plate in triplicate and incubated overnight at 4 °C. After three times washing with DPBST 0.05%, 50 μL of 1% BSA was added and incubated for 2 h at RT to block the unbound surface. After washing with DPBS, 50 μL of cell-derived exosomes (1.0 × 10^9/mL) or plasma exosomes (10× diluted with DPBS) were added and incubated at 4 °C overnight. After washing three times with DPBS, exosomes were treated sequentially with the following solutions: 50 μL of 2 μg/mL target-specific Abs (2 h, RT), 50 μL of the anti-mouse secondary Ab conjugated with horseradish peroxidase (HRP, 1:3400 dilution in 1% BSA, 2 h, RT), and 100 μL of TMB (30 min, RT), with three times washing using DPBS between steps. Oxidation of TMB was stopped with 100 μL of 2 M H2SO4. The optical density was measured at 450 nm using a BioTEK ELx800 microplate reader. Isotype IgG was replaced as the negative control for the primary Ab step.

2.5. Sample Preparation and Data Collection with DISVT

Samples were prepared following the developed methodology described in chapter 3. Briefly, A 4-inch Si wafer was coated with a thin layer of epoxy solution and then applied with a standard glass slide, followed by heating for 2 h at 150 °C. After cooling to RT, the glass slide was stripped from the wafer using a razor blade and metal tweezers, which led to a uniform and ultraflat Au film on the glass slide. Then, a black vinyl tape with multiple 5mm holes was applied to the Au surface to form a multi-well Au chamber slide. The Au chamber slide was then functionalized.
with the rabbit CD81 monoclonal Ab thiolated PEG linker by incubation. The optimized binding protocol used CD81-PEG-SH 1000 25 mg/mL in 5% BSA for 12 h at RT. After incubation, samples were washed with DPBST 0.01% three times and then rinsed with DPBS three times. The chamber slide was then saturated with hydrophilic but uncharged 0.1 mM MUTEG to block nonspecific interactions of the slide with biochemicals in subsequent steps and incubated for 1 h at RT. After incubation, samples were washed with DPBST 0.01% three times and then rinsed with DPBS three times.

Next, exosomes were captured (immobilized) on the Au slide by incubating low-concentration exosomes (∼10⁸ exosomes/mL) for 2 h at RT. The low concentration of exosomes is essential to allow detection. It also avoids the steric effect for binding AuNPs to ensure the detection of small exosomes. After incubation, samples were washed three times with DPBST 0.01% and rinsed with DPBS three times. Next, exosomes were labeled with the target-specific Ab-conjugated AuNPs by incubating the conjugates (50 pM in DPBST 0.05%) for 1 h at RT (flipping every 15 min to keep AuNPs from settling on the surface). After incubation, each well was washed three times with DPBST 0.05% and rinsed three with DPBS. Finally, exosomes were labeled with a Cy5 fluorophore for localization of all exosomes by incubating CLS-PEG-Cy5 2000 at 20 μM at 37 °C for 15 min, and each well was then rinsed with DPBS and water three times to remove any excess DPBS crystals and dried with nitrogen. DF/fluorescent images were then acquired using the dual imaging system and then analyzed using the SEDIA program.

2.6. Statistical Analysis

Statistical analysis was performed to compare the expression levels of targeted proteins on human subjects of different groups using ANOVA with the post hoc Scheffe method. A p-value ≤0.05 was considered significantly different. The mean difference between different groups was
considered significant if the absolute value was greater than the considerable minimum difference derived from the Scheffe method. In addition, the diagnostic value of HER2 in BC patients was evaluated by ROC curve analysis using R packages.

3. Results and Discussion

3.1. Characterization of Plasma Exosomes with NTA and SEM

NTA was used to measure the HD sizes of a healthy donor and patient exosomes. The size for healthy donor and patient exosomes ranged from 107 ± 35 to 189 ± 48 nm, respectively, with concentrations between $1 \times 10^9$ to $3 \times 10^{10}$ exosome/mL (Figure 5.1A&B). Analysis showed no statistical difference between size and concentration between patients and healthy donors. Figure 5.1C shows an SEM image of exosomes, which demonstrated the round to cup-shaped exosomes with sizes smaller than 200.

![Image](image.png)

**Figure 5.1:** (A) NTA of Healthy exosomes (B) NTA of Patient exosomes (C) SEM of exosomes.

3.2. HER2 Detection and Profiling of Plasma Exosomes with DISVT

To examine the clinical potential of our DISVT for the early detection of HER2-positive BC, we profiled HER2 expression on the plasma exosomes from HER2-positive BC patients at early stages ($n = 8$ for stage I and $n = 2$ for stage II) and the locally advanced stage (stage III, $n = 10$) in comparison to two controls, HER2-negative stage III patients ($n = 10$) and healthy donors ($n = 10$). Figures 5.2-5.5 show the HER2 expression profiles (the exosome population density histogram) from individual subjects for each cohort. For space reasons, we show nine subjects for...
Figure 5.2: Exosome population density histograms for healthy donors with HER2/AuNPs (red) and IgG/AuNPs (grey). Each graph represents one subject.

Figure 5.3: Exosome population density histograms for HER2-positive early-stage BC patients with HER2/AuNPs (red) and IgG/AuNPs (grey). Each graph represents one subject.
Figure 5.4: Exosome population density histograms for HER2-positive stage III BC patients with HER2/AuNPs (red) and IgG/AuNPs (grey). Each graph represents one subject.

Figure 5.5: Exosome population density histograms for HER2-negative stage III BC patients with HER2/AuNPs (red) and IgG/AuNPs (grey). Each graph represents one subject.
each group. The red graphs represent histograms obtained with HER2/AuNPs, and the grey graphs represent histograms obtained with IgG/AuNPs. It is evident that the stage III patients showed more HER2-positive exosomes than the stage I patients. Furthermore, The two controls did not show much difference between their HER2/AuNPs and IgG/AuNPs.

**Figure. 5.6A** shows the $F_{HER2}$ values of each subject in the four groups. The $F_{HER2}$ values for the healthy and HER2-negative control groups were lower than 3%. The $F_{HER2}$ values for the patients exhibit considerable heterogeneity, ranging from 3 to 17% for early-stage patients and 4 to 50% for stage III patients. The early-stage patients had an average of 9.5% HER2-positive exosomes, and the stage III patients had an average of 32.9% HER2-positive exosomes.

The data was statistically analyzed with ANOVA, with $p \leq 0.05$ significantly different. The results showed that the $F_{HER2}$ values of the early-stage and stage III patients were substantially different from that of the healthy control, with $p = 1.5 \times 10^{-3}$ for early-stage patients and $p = 1.0 \times 10^{-4}$ for stage III patients (**Figure 5.6B**). The $F_{HER2}$ of early-stage patients was also significantly different from that of stage III patients ($p = 2.1 \times 10^{-3}$), suggesting that our DISVT can detect cancer at early and locally advanced stages by quantifying the fraction of cancer marker-positive exosomes of the CD81-positive subtype. It can also differentiate the early stage from the locally advanced stage patient samples. There was no statistical difference between the healthy control and HER2-negative stage III patients ($p = 0.33$), indicating high specificity of DISVT for the detection of target cancer markers on plasma exosomes.
3.3. **HER2 Detection of Plasma Exosomes with ELISA**

For comparison, we further analyzed the plasma samples with ELISA. We measured HER2 expression level on the plasma exosomes from HER2-positive BC patients at early stages \((n = 10)\) and the locally advanced stage (stage III, \(n = 10\)) in comparison to healthy donors \((n = 10)\) (Figure 5.7A). Similar to DISVT, we used CD81 to capture exosomes in the ELISA analysis. The data was statistically analyzed with ANOVA, with \(p \leq 0.05\) being statistically different. The results showed that the absorbance from that of a significant difference between healthy and stage III patients \((p = 4.6 \times 10^{-3})\). However, there was no statistical difference observed between the healthy control and early-stage patients \((p = 0.057)\) or early-stage and stage III patients \((p = 0.24)\) (Figure 5.7B). This suggests that sandwich ELISA cannot detect early-stage, cancer-derived exosomes. Furthermore, this suggests that sandwich ELISA does not have high specificity for detecting target cancer markers on plasma exosomes.
3.4. *Comparison of DISVT and ELISA*

The diagnostic strength of exosomal HER2 expression for HER2-positive BC with DISVT was further compared to ELISA using ROC curves (Figure 5.8). The ROC curve shows an agreement with the ANOVA analysis that the DISVT was statistically more sensitive than ELISA in detecting; early-stage BC compared to healthy controls, with the area under the curve (AUC) = 0.99 for DISVT versus AUC = 0.73 for ELISA, healthy control compared to stage III BC, with AUC = 1 for DISVT versus AUC = 0.85 for ELISA. These studies demonstrate that DISVT profiling of exosomes can detect BC at early stages, is superior to the bulk ELISA that can only detect locally advanced BC.
Figure 5.8: Comparison of ROC curves between DISVT and ELISA for early cancer detection. (A) Healthy control versus early-stage BC, (B) healthy control versus stage III BC, (C) and early-stage versus stage III BC.183

The difference in the detection sensitivity between ELISA and DISVT is due to the difference in the marker detection and signal collection methodologies. The DISVT detects HER2 on individual exosomes, with the detection sensitivity down to a single molecule, as the DF image can detect AuNPs at the single particle level. It detects target-specific exosomes at the single vesicle level in the sea of normal exosomes that do not express HER2 proteins. However, ELISA detects collective HER2 signals from the bulk of exosomes. If the number of HER2-positive exosomes is low, the HER2 signal from these HER2-positive exosomes need to be higher to be detected by the ELISA plate reader. Thus, DISVT is superior in detecting rare disease signals in the presence of average backgrounds by identifying and detecting target-specific individual exosomes.

4. Conclusion and Future Outlooks

In conclusion, we have demonstrated that exosomal surface markers for the early detection of HER2 BC can be profiled and quantified using our DISVT system. In this method, we showed that DISVT was able to detect BC at early stages by profiling and quantifying using HER2 conjugated AuNPs on HER2-positive exosomes from the CD81-positive populations using DF and fluorescence imaging, using plasma samples from healthy donors and HER2-positive BC patients.
at the early and locally advanced stages. Furthermore, we have shown that our method can precisely and quantitatively detect HER2 surface protein markers on exosomes in plasma samples, thereby differentiating cancer exosomes from normal exosomes using cancer-associated surface protein markers. Late-stage patients show a factor of 13 times greater expression of HER2 than healthy donors, while early-stage patients show about five times higher HER2 expression than healthy donors. The high AUC value (AUC = 0.99) suggests that exosomal HER2 is a robust diagnostic marker for HER2-positive patients. In contrast, the sandwich ELISA bulk method failed to differentiate early-stage patients from both controls.

Compared to other SVT methods, our method is simple, rapid, and requires relatively low volumes of samples, following a straightforward capture, labeling, and measurement methodology, requiring about ~4 h total time if CD81 can be applied the night before. Detection was performed using DF and fluorescence imaging. Due to the advantages of simplicity, and high sensitivity, our method can be used in the clinical and research setting. However, the throughput at the current stage is also limited. Furthermore, this technique can be adapted to simultaneously analyze multiple samples using multi-well plates.

My colleagues and research collaborators will continue further testing and improvement of the DISVT. The next goal of this project is to demonstrate the efficacy and clinical potential of our SVT on stage IV plasma samples collected from BC patients, as well as increase cohort samples and markers for previously tested stages. The exosomes from HER2-positive breast plasma samples will be profiled for the cancer marker HER2, and results will be statistically compared with a profile of exosomes from healthy donors. The technology has the opportunity for further improvements, such as automation of sample deposition, processing, and image analysis, to ensure robustness and increase efficiency further. Exosome capture and labeling are performed manually,
which would be time-consuming when analyzing many clinical samples. Automation of sample preparation is entirely feasible, as we use a classic and straightforward capture and labeling strategy besides directly using a small amount of diluted biofluids. The eventual goal for our DISVT is to make it easily accessible for clinical standards and a benchmark for routine exosome analysis of various types of diseases.
Chapter 6: CONCLUSIONS

This dissertation has described the potential for using exosomes for protein detection and analysis. We have explained how exosomes could play a pivotal role in the development and advancement of cancer detection and monitoring. Exosomes are secreted by living cells with abundant biological information regarding their parent cells. This information can potentially be a valuable source for protein profiling of cancer biomarkers in liquid biopsies and the ability to better understand the roles exosomes play in cancer. However, challenges are present due to the small size of the exosomes, the low abundance of antigens on individual vesicles, and the complex biological matrix. Many recent studies have described advances in developing novel techniques using exosomes for liquid biopsies. However, the studies have fallen short due to needing specialized adaptations for standardized instrumentation to detect exosomes, keeping exosomal research in its infancy. More robust approaches must be developed before applying such technologies in the clinical setting.

Chapter 2 reviewed and summarized plasmonic nanotechnology-based devices and methods developed in past decades to detect and analyze molecular exosomes intended for cancer research and applications. Exosomes in body fluids have gained significant interest in developing new liquid biopsies for cancer diagnosis and monitoring. However, sensitive and specific molecular detection and analysis have ramifications due to their small size, low antigens, and complex biofluid matrix. Nanomaterials are widely used in developing protein and nucleic acid-based exosome detection and analysis, owing to the unique structure and functional properties of materials at the nanometer scale. Plasmonic nanomaterial using SPR, LSPR, SERS, colorimetric, electrochemical, and other technologies applications for exosome biomarker detection, profiling, and analysis were discussed.
In Chapter 3, we reported developing and optimizing our DISVT methodology for surface protein profiling of individual exosomes and quantifying target-specific exosome subtypes based on direct molecular capture of exosomes from diluted biofluids. The exosomes were first captured based on their CD81 expression. Next, surface proteins of interest were labeled using Ab-conjugated plasmonic AuNPs. Finally, all captured exosomes (cancerous and noncancerous) were labeled with an organic fluorophore to stain their lipid membrane. Darkfield microscopy was used to detect the light scattered from the exosomes labeled with AuNPs, followed by fluorescence microscopy to localize all CD81 capture exosomes. The Monte Carlo Method calculated the estimated AuNPs to detect exosomes down to 40 nm in diameter.

Chapter 4 reported the characterization of cell-derived BC exosomes using our DISVT method. We demonstrated our technology on exosomes derived from MDA-MB-231, SK-BR-3, and MCF-7 model cell lines, profiling them with EpCAM, HER2, and CD44 conjugated AuNPs and validated our results with conventional sandwich ELISA. Using MDA-MB-231, we found a high expression of CD44 with a low expression of HER2 and EpCAM ($F_p = 74\%, 5.0\%$, and $4.2\%$, respectively. Using SK-BR-3, we found a high expression of HER2 and EpCAM and a low expression of CD44 ($F_p = 78\%, 70\%$, and $5.0\%$, respectively). Finally, we used MCF-7 to find a high expression of EpCAM, a moderate expression of CD44, and a low expression of HER2 ($F_p = 75\%, 32\%$, and $2.3\%$, respectively). All of our findings closely resembled protein expression found using FC. With these findings, we found it feasible to move forward with exosomes derived from the plasma of HER2-positive and -negative BC patients of various stages and healthy donors.

In Chapter 5, we reported the potential of our DISVT method for the early detection of cancer using HER2-positive BC as a disease model. We profiled surface protein markers of interest across individual exosomes derived from plasma from healthy donors and BC patients. Using our
DISVT, but not the traditional bulk sandwich ELISA method, we showed the ability to detect HER2-positive BC at an early stage and differentiate between HER2-positive and -negative BC. We also showed that the amount of tumor-associated exosomes was a factor of three greater in locally advanced patients compared to early-stage patients, suggesting that single exosome surface protein profiling with DISVT can provide a suitable and high-sensitivity method for early cancer detection and quantitative monitoring.

Our SVT method can be further optimized, validated, and expanded using more markers and different cancer variants. This SVT has excellent potential to become a routine analytical technology for exosome content detection in fundamental exosome research or biomarker detection in clinical care.
REFERENCES

(1) National Institutes of Health (US). NIH Curriculum Supplement Series [Internet]. In Understanding Cancer; Biological Sciences Curriculum Study; National Institutes of Health (US): Bethesda (MD), 2007.


(21) Orrantia-Borunda, E.; Anchondo-Nuñez, P.; Acuña-Aguilar, L. E.; Gómez-Valles, F. O.; Ramírez-Valdespino, C. A. Subtypes of Breast Cancer. In Breast Cancer; Department of Medical Education, Dr. Kiran C. Patel College of Allopathic Medicine, Nova Southeastern


(36) Sancho-Albero, M.; Navascués, N.; Mendoza, G.; Sebastián, V.; Arruebo, M.; Martín-Duque, P.; Santamaría, J. Exosome Origin Determines Cell Targeting and the Transfer of


(115) Chao Li; Da-Ren Liu; Guo-Gang Li; Hou-Hong Wang; Xiao-Wen Li; Wei Zhang; Yu-Lian Wu; Li Chen. CD97 Promotes Gastric Cancer Cell Proliferation and Invasion through Exosome-Mediated MAPK Signaling Pathway. *WJG* 2015, 21 (20), 6215. https://doi.org/10.3748/wjg.v21.i20.6215.


(120) Sakamoto, S.; Patalun, W.; Vimolmangkang, S.; Phoolcharoen, W.; Shoyama, Y.; Tanaka, H.; Morimoto, S. Enzyme-Linked Immunosorbent Assay for the Quantitative/Qualitative


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Author: Jongmin Park, Hyungsoon Im, Seonki Hong, et al
Publication: ACS Photonics
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Date: Feb 1, 2018

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Author: Jaena Park, Miyeon Hwang, ByeongHyeon Choi, et al
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Dual Imaging Single Vesicle Surface Protein Profiling and Early Cancer Detection

Author: Kristopher Amrhein, Mitchell Lee Taylor, Raymond Wilson, et al
Publication: Applied Materials
Publisher: American Chemical Society
Date: Jan 1, 2023

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