Extracellular vesicles as a promising diagnostics platform for cancer

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1st Master of Science in Drug Development

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ABSTRACT

Extracellular vesicles (EVs), including microvesicles, exosomes and apoptotic bodies, are secreted by many, if not all, cell types. Through transfer of their molecular cargo and altering the properties of the recipient cells, EVs play an important role in the intercellular communication. The fact that tumor cells also release EVs raises opportunities to exploit these EVs as a diagnostic and prognostic tool for cancer and other pathologies. Although a lot still remains unclear concerning EV biogenesis and function, promising detection and analysis techniques are being developed to gain further knowledge in the potential of these EVs. In this paper, various key features concerning detection and analysis of tumor-derived vesicles are discussed and new methods are implemented in order to find potential tumor markers displayed on EVs.

Through a series of experiments, it was attempted to elucidate the relation between tumor marker expression on various tumor cells and their derived EVs. Correlation was observed concerning the expression of various tumor markers, e.g. EGFR, between both A431 and MDA-MB-468 tumor cells and their derived vesicles. ELISA, Western Blotting and immunocapture bead assays in combination with flow cytometry are several techniques used to expose this correlation. Moreover, optimization of existing techniques especially regarding EVs immunocapture bead assays was attempted as well as developing a new detection method. The presence of different subpopulations of EVs was demonstrated through preparation and usage of a different type of bead in this case; anti-EGFR beads.

A promising new detection method was used that can possibly exclude the use of expensive detection antibodies. 5-aminolevulinic acid (5-ALA) is a biochemical intermediate in the mitochondrial porphyrin pathway. It is converted mitochondrial into protoporphyrin IX (PpIX) which has intrinsic fluorescent capabilities. This paper aims on detection of this PpIX in EVs derived from A431 tumor cells using flow cytometry. Although the results are not clear-cut, further development and optimization may lead to promising results. All experimental data together enabled us to create a spectrum of different biomarker present on certain types of cells and vesicles. This data is essential to use EVs as a platform to develop promising diagnostic tools for cancer and cancer research in the future.
SAMENVATTING

Extracellulaire vesikels (EVs) bestaande uit microvesikels, exosomes en apoptotische lichamen, worden uitgescheiden door veel, al dan niet alle, celtypes. Deze EVs blijken een rol te spelen in de intercellulaire communicatie door transfer van hun moleculaire inhoud en de eigenschap om de functie van de ontvangende cel te modiferen. Het feit dat tumorcellen ook EVs uitscheiden heeft geleid tot een stijgende interesse om deze EVs te exploiteren als zijnde een diagnostisch en prognostisch hulpmiddel voor kanker en andere ziektes. Alhoewel er nog veel onduidelijkheid is omtrent EV biogenese en functie, zijn hoopgevende detectie en analyse technieken in ontwikkeling om meer kennis te vergaren inzake het potentieel van deze EVs. In deze paper worden verscheidene sleutelpunten aangehaald betreffende de detectie en analyse van tumor afgeleide EVs. Ook worden nieuwe methodes ontwikkeld om eventuele potentiële tumor merkers op deze EVs te ontdekken.

Door het uitvoeren van experimenten is getracht de relatie tussen cellen en hun EVs inzake tumor marker expressie op te helderen. Correlatie werd gevonden inzake tumor marker expressie (EGFR) tussen A431 en MDA-MB-468 cellen en hun EVs. ELISA, Western Blotting en immunocapture beads assays in combinatie met flow cytometrie zijn verschillende technieken die gebruikt werden om deze correlatie aan te tonen. Bovendien werden bestaande technieken geoptimaliseerd en nieuwe detectiemethoden onderzocht. De aanwezigheid van verschillende subpopulaties van EV werd aangetoond door middel van het gebruik van nieuwe gefabriceerde anti-EGFR beads.

Een veelbelovende methode werd gebruikt die eventueel het gebruik van dure fluorescente antilichamen kan uitsluiten. 5-aminolevuline zuur (5-ALA) is een biochemisch intermediair in de mitochondriale porfyrine pathway. 5-ALA wordt omgezet in de mitochondriën tot het fluorescente protoporfyrine IX (PpIX). Deze paper beoogt om dit PpIX te detecteren in EVs van de A431 tumor cellijn via flow cytometry. Alhoewel de gevonden resultaten niet eenduidig zijn, kan verdere optimalisatie en ontwikkeling van deze techniek leiden tot veelbelovende resultaten. Deze data gebundeld stelt ons in de mogelijkheid om een spectrum te creëren aan verscheidene biomarkers aanwezig op tumor cellen en hun EVs. In de toekomst kan deze data van groot belang zijn om EVs te gebruiken als diagnostisch platform voor kanker en kanker onderzoek.
ACKNOWLEDGEMENTS

Firstly, I would like to thank Prof. dr. K. Braeckmans for giving me the opportunity to experience life and research work abroad.

This thesis would not have been possible without the time, dedication and both intellectual as emotional support of my supervisor Dr. Roy van der Meel. I would like to use this opportunity to thank him for his kindness and for his ever so optimistic vision.

I would like to thank Prof. dr. Raymond Schiffelers and the Laboratory of Clinical Chemistry and Hematology of UMC Utrecht for the helpful advice and the daily support.

Finally, I would like to thank my girlfriend, my parents, my friends and brother for the love, confidence and support during the writing process of this thesis
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-ALA</td>
<td>5-aminolevulinic acid</td>
</tr>
<tr>
<td>1 G</td>
<td>1 gravitational unit or $6,67384 \times 10^{-11} , \text{m}^3 , \text{kg}^{-1} , \text{s}^{-2}$</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celcius</td>
</tr>
<tr>
<td>µL</td>
<td>Micro liter</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin Channel</td>
</tr>
<tr>
<td>aqua dest.</td>
<td>Distillated water</td>
</tr>
<tr>
<td>BO</td>
<td>Beads Only</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CO</td>
<td>Cells Only</td>
</tr>
<tr>
<td>Da</td>
<td>Unified atomic mass unit</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>EpCAM</td>
<td>Epithelial Cell Adhesion Molecule</td>
</tr>
<tr>
<td>EVs</td>
<td>Extracellular Vesicles</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward Scattering</td>
</tr>
<tr>
<td>HRP</td>
<td>Streptavidin-multi horseradish protease</td>
</tr>
<tr>
<td>ISO</td>
<td>Isotype control antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro ribonucleic acid</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular Body</td>
</tr>
<tr>
<td>Nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NTA</td>
<td>Nanoparticle Tracking Analysis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PE</td>
<td>R-phycoerythrin</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplying Tubes</td>
</tr>
<tr>
<td>PpIX</td>
<td>Protophorphyrin IX</td>
</tr>
<tr>
<td>PS</td>
<td>Penicillin/streptomycin</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>RPM</td>
<td>Rounds Per Minutes</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SSC</td>
<td>Side Scattering</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>T/E</td>
<td>Trypsine/Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming Growth Factor α</td>
</tr>
<tr>
<td>V</td>
<td>Voltage</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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1. INTRODUCTION

Organisms are made up of many living cells. These cells allow us to grow as they divide and differentiate into cells with a specific function. However, sometimes this mechanism can be disturbed. Cancer is a collective term for diseases that are characterized by uncontrollable cell growth leading in most cases to the formation of a tumor. These tumors can invade or crowd normal tissue leading to life threatening situations.

An important role in the tumorigenesis of multiple cancer types is played by the epidermal growth factor receptor (EGFR), a growth factor receptor tyrosine kinase, and its ligands such as epidermal growth factor (EGF) and transforming growth factor α (TGF-α). In certain epithelial cancer types such as ovarian, cervical, epidermal and oesophageal cancer, activation of EGFR by its ligands can be the driving force for proliferation and differentiation of cells and ultimately leading to critical growth behavior of cancer cells. In healthy cells, growth factor receptor signaling is a controlled process that contributes to cell proliferation. In cancer cells, however, growth factor signaling can be disturbed leading to uncontrollable growth. Elevated EGFR expression can be a strong prognostic factor for certain tumor types and inhibition of EGFR signaling has led to significant therapeutic benefits in the healthcare.1,2,2–4 Erlotinib (Tarceva®), a tyrosine kinase inhibitor, and cetuximab (Erbitux®), a humanized monoclonal antibody are examples of targeted anti-EGFR drugs for treatment of cancer, with several others in the clinic or under development4.

Another important element in cancer biology is cell-to-cell communication. Cells exchange information via direct contact or by secretion of soluble compounds. A third way of cell-to-cell communication is mediated by membrane-derived vesicles, called extracellular vesicles (EVs). EVs are released in the extracellular environment by many, if not, all human cell types. These secreted vesicles reach the bodily fluids and circulate through the whole human body, providing opportunities for EVs to interact with a vast amount of different cells. Communication between cells and vesicles occurs through transmitting of signals by either direct interaction between the vesicle membrane protein and the recipient membrane protein5,6 or by internalization of the vesicle content by the recipient cell.7,8 This enhanced interplay between vesicle membrane proteins and recipient membrane proteins makes EVs excellent natural drug delivery systems. In relation to cancer, EVs released by tumor cells
may have an impact on tumor angiogenesis\textsuperscript{9}, tumor invasiveness\textsuperscript{10} and on metastasis.\textsuperscript{11} The last decade there is a growing interest in vesicle analysis as a promising platform for biomarker development. The fact that the amount of secreted vesicles and their composition varies depending on possible pathological conditions conforms this interest. EV-based cancer screening opens new diagnostic and therapeutic opportunities and can provide big advantages especially in the differentiation between different tumor types using just a sample of bodily fluids such as blood, saliva and urine. It provides remote, direct and noninvasive information on cancer cells such as therapeutic targets, different tumor entities, genetic material, etc. As EVs provide a fingerprint of their parent cell, the isolation and analysis of EVs from biofluids can be considered a 'liquid biopsy'.\textsuperscript{6,12}

Nowadays, the process of taking a biopsy can be a very invasive intervention e.g. in case of a brain tumor. Also, in most cases a biopsy is taken when the patient has already reached a critical stadium of tumorigenesis and often the tumor has metastasized. Therefore, EV screening can potentially become a new routine, noninvasive and straightforward method to detect cancer even before initial symptoms appear. However, standardizing the identification and quantification of vesicles is needed in order to apply EV detection for diagnostic and/or prognostic purposes. Regarding the standardization, it is important to notice that EV’s obtained from clinical samples are likely to be very heterogeneous and arose from different biological processes in the human body.\textsuperscript{13}

1.1 THE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

The human epidermal growth factor receptor (EGFR) family consists of four tyrosine kinase receptors: ErbB-1/EGFR, ErbB-2/HER2, ErbB-3/HER3 and ErbB-4/HER4. This family of receptor protein kinases induces complex signal transduction pathways eventually leading to morphogenesis and to modulation of cellular responses such as cell proliferation, migration, differentiation, adhesion and migration. These transmembrane receptors consists of an extracellular ligand binding site, a transmembrane region and a cytoplasmic domain. This construction allows the receptor to transmit signals across the plasma membrane to activate gene expression and induce cellular responses such as proliferation and differentiation.\textsuperscript{1,2,4}
EGFR is activated through a series of ligands such as epidermal growth factor-like (EGF) ligands, transforming growth factor (TGF)-α and neuregulins. These ligands bind to the extracellular domain of the receptor and induce dimerization and cross-phosphorylation resulting in the formation of receptor homodimers. This cross-phosphorylation of tyrosine residues between an EGFR pair forms a transient receptor complex. Dissociation of the signaling complex leads to the release of activated proteins in the cytoplasm. These activated proteins induce different complex signal transduction pathways such as the phosphoinositol kinase pathway, the mitogen-activated protein kinase (MAPK) pathway, the antiapoptotic kinase Akt pathway and several transcriptional regulators.¹²

Growth factor signaling is essential for many physiological cellular processes and morphogenesis, but aberrant EGFR pathway signaling is also involved in the development and growth of tumor cells. Overexpression of EGFR is common in many of the most frequent human epithelial cancers. Decreased activity of EGFR leads to downregulation of these signaling cascades resulting in apoptosis of cancer cells. In terms of diagnostics, these receptors can be used as a tumor marker not only on tumor cells themselves but also on the vesicles they release.²¹⁴

1.2 EXTRACELLULAR VESICLES (EVs)

1.2.1. Biogenesis and secretion

Cell-to-cell communication is essential for multicellular organisms. Many human cells release membrane-derived vesicles which can influence adjacent and remote cells. Importantly, EV release has also been observed for cancer cell lines. At first EVs were thought of as cell waste without any specified function. Later on however, vesicles were identified to play a certain role in adaptive immune responses⁵. Since then there is a growing inquisitiveness regarding the specific function of these vesicles. EVs are thought to have an important regulating function in common fundamental physiological processes such as communication¹⁵, tissue repair¹⁶, blood coagulation¹⁷ and immunity⁵, but also in the development and progression of pathological circumstances such as cancer¹⁸,¹⁹, immunomodulation²⁰ and other
diseases. Shedding of EVs is supposed to be a physiological phenomenon that gives rise to cell activation and growth. Shedding is stimulated by different factors including hypoxia, oxidative stress and exposure to shear stress. 

It has become a fact that different types of vesicles co-exist, with different intracellular origins, biogenesis and/or surface markers and therefore probably different compositions and functions in the body. EVs is a general term used for different subtypes of vesicles which differ from each other in their cell origin, biological function, or biogenesis. The three main classes of EVs are microvesicles or ectosomes, exosomes and apoptotic bodies (Figure 1.1). The common characteristics that unifies the different classes is the fact that they are all small cell derived, lipid bilayer enclosed entities ranging from 30 nm to 2 µm in diameter. Due to the lack of consensus regarding the characterization, purification and biogenesis of vesicles, the term ‘extracellular vesicles’ is an overarching term used in many reviews, in articles and also in this paper.

1.2.2. Subtypes of extracellular vesicles

**Microvesicles** arise through outward budding and fission of the plasma membrane as a result of an alternation between cytoskeletal protein contraction and phospholipid redistribution. Activity of aminophospholipid translocase translocates phosphatidylserine from the inner leaflet of the plasma membrane to the outer leaflet and induces budding of the plasma membrane. These vesicles are usually bigger than the exosomes and have a size ranging from around 50 nm to 2000 nm.

In contrast to microvesicles which arise through outward budding of the plasma membrane, **exosomes** are a group of vesicles, traditionally thought to be derived from the endolysosomal pathway. They are formed in a so called endosomal network which is a membranous system that sorts the various intraluminal vesicles (ILVs), lysosomes and cell surface membranes and controls their destination. There are 3 subtypes of endosomes; early endosomes, late endosomes and recycling endosomes. Early endosomes fuse with endocytic vesicles and incorporate their
content destined for recycling, degradation or exocytosis. Content that has to be recycled is sorted in the recycling vesicles. The remaining early vesicles undergo multiple transformation to eventually become late endosomes. During these transformations content which is destined to be exported or degraded tends to bud inwards and form ILVs with a size usually between 40 and 100 nm. Therefore, these late endosomes accompanied by their multiple small ILVs are also called multivesicular bodies (MVBs). Studies have shown that the formation of ILVs is regulated by endosomal sorting complex required for transport (ESCRT)-components and its associated proteins, and also by an ESCRT-independent machinery, activated by G-protein coupled sphingosine 1-phosphate. Secretion of these vesicles in the extracellular environment can occur through fusing of the MVB with the plasma membrane and which is controlled by various Ras GTPases. These secreted vesicles are called exosomes. Exosomes have a relatively homogenous size distribution and due to their endocytic origin, they can contain proteins involved in the endolysosomal sorting process as cargo. These proteins can serve as exosomal markers. The size range of microvesicles and exosomes overlaps, therefore the difference in biogenesis remains the parameter to distinct between the two groups. 6,24,26,27

Due to the fact that microvesicles are derived from outward budding of the plasma membrane it seems that they mirror the membrane structure of the parent cell more accurately than exosomes do. Both subtypes of EVs contain cytoplasmic proteins, certain membrane surface proteins and RNA’s but due to the specific biogenesis of exosomes they carry additional components such as tetraspanins (CD9, CD81 or/and CD63). These tetraspanins are a family of membrane proteins most commonly associated with EVs and are mainly used as EV markers. 26,36,37

Apoptotic bodies are generally larger than exosomes and microvesicles and have a size ranging from 50 nm to 4000 nm. Contrary to the biogenesis of exosomes and microvesicles, apoptotic bodies are formed when a cell undergoes apoptosis. The process of apoptosis is characterized by different stages that begins with condensation of the nuclear chromatin, weakening of the cell membrane followed by processing and packing of the cellular content in membrane enclosed entities
named apoptotic bodies. These vesicles accommodate DNA, histones and organelles. Specific interaction between certain receptors on phagocytes and the changed membrane composition leads to phagocytosis of the apoptotic bodies, thereby preventing dissipation of the cell content in the extracellular environment.\textsuperscript{6,26}

Figure 1.1: Different subtypes of EVs released by cells. Exosomes are released through fusion of the MVB, generated by inward budding of the early endosome, with the plasma membrane. Microvesicles, usually bigger than exosomes, arise through outward budding and fission of the plasma membrane. Apoptotic bodies are released through random blebbing of the plasma membrane when cells undergo apoptosis. Vader \textit{et al.}\textsuperscript{25}

1.2.3. Extracellular vesicles as a communication vehicle

Cell-to-cell communication is an important factor for proper coordination during development and growth of different cell types within adult tissues. Cells are known to communicate through secreting soluble factors and direct cell-to-cell contact. Additionally, EVs released by certain if not all cell types represent another mediator of cell-to-cell communication. It provides a new understanding of signal and molecule transfers between cells both over remote and short distances.\textsuperscript{38,39}
After release, EVs achieve their biological effects through interacting with the recipient cells in three different ways. Firstly, direct interaction can take place through fusing of the EVs with the target cell and transfer of their cargo. Interaction can also take place through endocytotic uptake and release of their cargo. Lastly, EVs may activate cell signaling through ligand/receptor interactions with the target cell (Figure 1.2). When internalization takes place, EVs can fuse with endosome membranes and release their content in the cytoplasm.\textsuperscript{40–42} The function of EV depends on their parental cell and their cargo. They can act as signaling complexes during development, transfer membrane receptors to target cells and pass on genetic information through horizontal transfer of RNAs and thereby induce modification of the receiving cell.\textsuperscript{15,41,43–47}

![Diagram showing interaction of EVs with the recipient cell](image)

**Figure 1.2:** Interaction of EVs with the recipient cell occurs in three possible ways. EVs can achieve their biological effects through fusing with the target cell and releasing their content or may activate cell signaling pathways by ligand/receptor interactions. Interaction can also take place through endocytotic uptake and release of their cargo. Lastly, EVs can also release their cargo into the extracellular space. Turturici et al.\textsuperscript{15}
1.2.4. Extracellular vesicles as mediators of tumor progression

EV cargo may include proteases, adhesion molecules, signaling molecules, DNA, mRNA, and microRNA sequences.\textsuperscript{34} However, controversy arose from the fact that these EVs can also transfer active molecules responsible for tumor progression and metastasis also known as oncoproteins, such as EGFR and its constitutively active mutant EGFR\textsuperscript{vIII}.\textsuperscript{4,25,34}

The formation of vesicles is linked with tumor progression in three ways: through the mechanism of EV generation, through EV content and through the participation of EVs in various tumor-related processes such as angiogenesis, tumor invasion and metastasis.\textsuperscript{34} However, a lot remains unclear about the specific role and biogenesis of oncogene-containing EVs, also called oncosomes, in cancer progression. Recent studies have shown that EV production can be triggered by the activity of the same oncogenic receptors (e.g. EGFR) that afterwards become incorporated into the EV cargo. It is reasonable that these vesicles transfer their oncogenic content into recipient cells and potentially change the cells properties, thereby activating complex signal transduction pathways such as the MAPK and the AKT pathways and expressing VEGF inducing tumor growth and angiogenesis. These and other effects identify tumor-derived EVs as possible mediators of oncogenic and proangiogenic effects\textsuperscript{34,50} and are therefore considered as an inducer of tumor in normal recipient cells.

1.2.5. Extracellular vesicles as a diagnostics platform

Early detection of cancer is crucial to reduce mortality and increase survival. Hence it is essential to seek for biomarkers to discriminate healthy individuals from cancer patients. Proteins derived from solid tumor types that are found in bodily fluids, especially blood, can function as biomarkers for a disease, such as prostate-specific antigen for prostate cancer. A downside of these biomarkers is the lack of specificity.\textsuperscript{51} For example, an increased prostate-specific antigen can also be linked to benign prostate hyperplasic conditions.\textsuperscript{51,52}
EVs have generated considerable excitement in cancer research as potentially novel noninvasive biomarkers which can affirm the outcome of the currently accepted biomarkers. EV-based diagnostics are expected to be less invasive compared to standard biopsies as they are present in the bodily fluids. Cancer cell-derived EVs have been studied extensively in order to elucidate their potential as a diagnostic and prognostic tool. EVs carry specific molecular markers typical for the cells of their origin e.g. epithelial cell adhesion molecule (EpCAM), epithelial growth factor receptor (EGFR), human epidermal growth receptor 2 (HER-2), vascular endothelial growth factor (VEGF), metalloproteases, and many others which contribute to the suitability of these vesicles as disease biomarkers. In addition to the overexpression of biomarkers, elevated presence of EVs in case of a tumor also contributes to the fact to exploit these EVs as a potential diagnostic tool. A challenge with these vesicles is a lack of specificity due to overlapping surface markers on the vesicles of different kinds of tumors.

Beside the presence of specific molecular markers, EVs can also contain nucleic acids including mRNAs and microRNAs. The functionality of these nucleic acids contributes to the cell-to-cell communication but also seem to be a promising cancer biomarker. Recent reports discussed that these microRNAs and mRNAs are potentially involved in the in vitro enhancement of angiogenesis and endothelial cell proliferation. It has been reported that circulating tumor associated microRNAs in blood can serve as cancer biomarker. In addition, EVs can contribute to the diagnostic strength of these circulating miRNAs as discussed by Taylor et al. The authors isolated EVs expressing EpCAM, a surface protein which is overexpressed in ovarian cancer, from the serum of ovarian cancer patients. Elevated levels of EpCAM positive vesicles were seen within the ovarian cancer patients. Additionally, EVs microRNAs profiles from ovarian cancer patients were significantly different to the profiles found in patients with the benign form. This suggests that analyzing a combination of nucleic acids and surface proteins form tumor derived EVs offers an opportunity to overcome the lack of specificity seen when analyzing just surface markers on the tumor derived EVs. Promisingly, Sun et al. and Li et
al.\textsuperscript{64} showed that a combination of EV isolation and microRNA array might be a suitable method to differentiate between healthy individuals and cancer patients.

There is a great interest in using EVs as a novel diagnostic tool and even as a therapeutic tool, despite that many aspects of EV biology still need to be elucidated. Further research is needed to elucidate the function, composition and biology of EVs in order to develop accurate cancer diagnostic method needed and explore novel therapeutic strategies.

1.2.6. Potential therapeutic applications of EVs

EVs trafficking in certain types of tumors can have an influence on tumor metastasis, angiogenesis and invasiveness implying the potential therapeutic benefit achieved when blocking this particular EVs trafficking. Interfering with EVs biogenesis can be an option to block cancerous EV production by cancer cells and has been described by Peinando \textit{et al.}\textsuperscript{18}. On the other hand as shown by Al-Nedawi \textit{et al.}\textsuperscript{65}, EV uptake by recipient cells can be inhibited through blocking of their surface receptors with Annexin V, preventing the interaction between vesicles and surface receptors on the target cells. Another possible way to decrease EV trafficking is to eliminate tumor-derived EVs from the circulation.\textsuperscript{66}

Previous potential therapeutic methods are based on the blockage of EV trafficking but it should be mentioned that EV release and uptake can also be beneficial. EVs can, for example, be used as natural drug delivery systems to bring therapeutic compounds such as tumor suppressive drugs across the tissue barriers.\textsuperscript{67} It is, however, important to notice that these all are hypotheses which may become novel anti-cancer therapies in the future.

1.3 CHARACTERIZATION OF EVs

It is essential to determine EV concentration and content in order to develop a possible diagnostic assay and/or to find an appropriate therapeutic treatment. Nowadays, there are several different techniques to determine size distribution, concentration, proteins and nucleic acid contents of EVs.
1.3.1. Size and concentration of EVs

Determination of EV concentration and size distribution in a biological sample is not straightforward. EVs are of nanosize, challenging to visualize and require specific equipment and methods. A common technique is nanoparticle tracking analysis (NTA). This technique involves a monochromatic laser light source to illuminate nanoparticles with a size ranging from 10 to 1000 nm. EVs are seen as vibrating individual scattering points moving under Brownian motion. Frame by frame videos of around 60 seconds are recorded via a microscope equipped with a camera and analyzed with the NTA software. The path described of each vesicle in frame in function of time determines their velocity which can be used to calculate their size by applying the two dimensional Stokes-Einstein equation. The included NTA software processes this information, automatically calculates the particle size and gives an estimation of the particle concentration in the applied sample. Other possible techniques such as transmission electron microscopy, direct flow cytometry and resistive pulse sensing are not used in this paper.

1.3.2. Immunocapture bead assay

1.3.2.1. Magnetic immunocapture beads

Magnetic beads are used as a tool to study and compare different populations of EVs released by cells. These beads usually have a diameter of around 3 µm and are coated with antibodies that recognize EV surface proteins. They are optimized for use with cell culture supernatant. This allows enrichment and isolation of certain vesicle subpopulations depending on the kind of coated antibody, e.g. tetraspanins CD9, CD63 and CD81 (Figure 1.4).

1.3.2.2. Flow cytometry

Flow cytometry is a widely used technique to analyze cells and EVs. It consists of a laser, light scattering and fluorescence detectors, a hydrodynamic focusing tube, sheath fluid and software. As a sample suspension is introduced into the machine, the different particles are hydrodynamically focused using sheath fluid through a
small nozzle resulting in a stream of single particles. These particles or cells pass the laser one by one and scatter light which is picked up as forward scattering (FSC) and side scattering (SSC) by different detectors. These different directions of scattering gives an image of particle size and density whereby FSC correlates with particle size and SSC with particle density. The software processes this information and formulate a dot plot of the SSC in function of the FSC. Based on this plot it is possible to distinguish between different subpopulations of particles/cells as each particle or cell subpopulation has its own size and density thus its own SSC and FSC. Besides these detectors, flow cytometers also consist of a fluorescence detector which allows to measure particles or cells with a fluorescent label, e.g. an antibody conjugated with a fluorochrome. This fluorochrome is excited by the laser and emits light with a different wavelength. Via the use of mirrors and filters, sensors called photomultiplying tubes (PMTs) will detect fluorescence at a specified wavelength. The PMTs convert the energy of the emitted photons into an electrical pulse expressed as a voltage. These measured voltage pulses or so called events are processed by the software and plotted in function of the intensity of fluorescence or channel number for that particular event (log scale). This latter method using flow cytometry in combination with fluorescent labelled antibodies to sort cell populations is also called fluorescence-activated cell sorting flow cytometry (FACS)74,75 (Figure 1.3).
Hydrodynamic focusing leads to a tiny stream of individual particles passing the laser beam. As a result these particles scatter light in several directions which is picked up by FSC and SSC detectors. Particles conjugated with a fluorochrome emits light with a certain wavelength which is focused by mirrors and filters onto the PMTs.\footnote{b}

Due to their small size, EVs are difficult to analyze with flow cytometry which usually detects cells ranging from 500 nm to 50 µm.\footnote{c} After incubation of immunobeads with EVs, these complexes are incubated with specific fluorescent antibodies against biomarkers on the surface of the EVs making them useful for FACS flow cytometry (Figure 1.4).\footnote{73} This method however is a semi-quantitative method which results in a mean fluorescence intensity of the EV coupled beads which correlates with the amount of detected surface marker on the vesicle and not an absolute number of vesicles with the specific surface marker.

Figure 1.4: Immunocapture and analysis of EVs. Magnetic iron bead coated with CD9, CD63 or CD81 antibodies captures and enriches EVs with the same surface proteins. The second image represents the bead-EV-fluorescent antibody complex used for FACS flow cytometry.\footnote{d}

1.3.3. Enzyme-linked immunosorbent assay (ELISA)

Another method for the characterization of EVs is an ELISA. Briefly, EVs are incubated with a coated surface, usually a well plate, followed by an incubation with detection antibodies linked to an enzyme, e.g. horseradish peroxidase. This
enzyme converts the substrate to a luminescent compound of which the signal is measured and correlates with the expressed surface proteins. Using this assay it may be possible to capture the subpopulation of EVs that expresses a certain surface or tumor marker such as EGFR or CD9.

1.3.4. Western Blotting

Western Blotting can be used to detect proteins of interest in cell or EV lysates. Firstly, gel electrophoresis is performed prior to the actual Western Blotting. During gel electrophoresis, cell and vesicle lysates are brought on a gel and proteins within the lysates are separated based on their size during migration over a polyacrylamide gel under influence of an electric field supplied by a power source. Sodium dodecyl sulfate (SDS) is added to denaturize the proteins becoming long strands which experience an even greater resistance passing through the gel. Moreover, SDS causes a negative charge to form around the already existing charge of the proteins making them migrate to the opposite pole, the positive pole.

The acquired gel with the separated proteins is then transferred to a membrane. On this membrane, which is an exact copy of the polyacrylamide gel, detection antibodies can be added to image the separated proteins from the examined cells and vesicles lysates. Using this assay, an image can be created about the total amount of a specific protein in a cell or vesicle lysate. This allows to gain further information about the relationship between cells and their vesicles.

1.4 CELL CULTURE

Most tumor cell lines came into existence in the 70’s as taking a biopsy and culturing cells became much more efficient. Culturing these different tumor cell lines can give an insight in which specific type of receptors and proteins the tumor overexpresses. Also the medium of the cultured cells can be analyzed in search for derived vesicles and their possible surface and/or tumor markers in order to find a diagnostic assay or even an appropriate therapeutic treatment.
1.4.1. A-431 cancer cell line

The A-431 cell line is one of the cell lines confirmed by Giard *et al.*\(^7^9\) It is an epidermoid carcinoma cell line derived from a solid squamous carcinoma of an 85-year-old female. Not only the A-431 cells are known to overexpress EGFR, but also their secreted vesicles contain the EGFR on their surface.\(^8^0,8^1\)

1.4.2. MDA-MB-468 cancer cell line

The MDA-MB-468 cell line was established in 1977 by R. Cailleau *et al.*\(^8^2\) It is a metastatic breast adenocarcinoma derived from a pleural effusion of a 51-year-old black female patient. MDA-MB-468 cells secrete vesicles and overexpress EGFR suggesting the fact that the vesicles derived from this cell line also display EGFR on their surfaces.\(^8^3\)

1.5 5-AMINOLEVULINIC ACID (5-ALA)

5-Aminolevulinic acid (5-ALA) is a biochemical intermediate in the mitochondrial porphyrin pathway which leads to the formation of heme. It is metabolized into protoporphyrin IX (PpIX), an endogenous fluorophore normally secreted by human tissue. In cancerous tissue however, there is an excess of intracellular PpIX produced causing the product to accumulate.\(^8^4\) This results in elevated fluorescent tissue when illumination occurs at the right absorption wavelength. The excitation peak of PpIX is reached at around 405 nm and leads to red fluorescent emission, peaking at 635 nm.\(^8^5\)

5-ALA is an EMA approved orally administered drug marketed as Gliolan\(^\text{®}\) and used as an extrinsic fluorescent contrast agent in fluorescence image-guided surgery (FIGS). Based on the difference in accumulation of the endogenous fluorophore PpIX between cancerous and normal tissue, detection and analysis by fluorescence becomes more efficient to discriminate between premalignant/malignant tissue and healthy tissues. During an operation, enhanced visual difference between different tissues can lead to adequate visualization of the malignant tissue and thereby decreasing morbidity and surgical duration.\(^8^5\)
In terms of diagnostics, it may be feasible to detect this fluorescence after oral application in tumor-derived EVs suggesting that the fluorescent PpIX is incorporated in the cargo of the vesicles. A main advantage may be that there is no need for expensive detection antibodies. The fluorescent vesicles can then be subjected to an examination to elucidate the tumor specific surface markers and try to discover from which tumor the vesicles are derived from.
2. OBJECTIVES

The last decade, there is a growing interest in the biology and biogenesis of EVs in order to use them as a therapeutic vehicle, or to exploit them as a diagnostic tool for cancer and other pathologies. Cell-derived EVs serve multiple critical biologic functions including intracellular communication and modulation of the tumor microenvironment. Because EVs contain genetic and proteomic contents that mirror the cell of origin, it is possible to detect tumor-specific biomarkers or proteins in or on EVs secreted by tumor cells. Until now, no consensus is yet achieved concerning the relation between EVs and their parent cells. Therefore, it is necessary to enlighten potential biomarkers on these vesicles and reach a standardized process of EV detection, quantification and analysis.

In this paper, key features in EV immunodetection are tested and justified. Antibody concentration, detection linearity, EVs storage, different degrees of filtration, different amounts of used cell medium and different beads are only a small part of the hugely variable clinical and research aspects present. These are important variables as they can reduce the cost of an assay, e.g. by using a less concentrated antibody solution or less working pressure, e.g. by storage of EVs samples in the fridge. Regarding 5-ALA, new methods for the detection of EVs were used and analyzed. A431 and MDA-MB-486 cells and their derived EVs were examined using multiple bead assays, Western Blotting and ELISA’s.

The main emphasis however, lays on the detection of biomarkers on cells and cell-derived EVs and the relation between the two. This enables us to create a spectrum of different biomarkers present on certain types of cells and vesicles. In the near future, this data is essential for the development of a promising and non-invasive diagnostic tool based on EVs.
3. MATERIALS AND METHODS

In order to find an appropriate assay for the detection of vesicle/tumor surface markers, it is required to culture tumor cell lines in order to obtain the shed vesicles. During growth, cultured tumor cells will shed vesicles in the medium due to several factors such as stress, hypoxia and many others as discussed above. Also the amount of vesicles depends on the culturing conditions such as incubation period, the amount of cells and cell type. Thereafter, secreted vesicles can be separated from the cells and used for experiments.

3.1 CELL GROWTH AND VESICULATION

A431 and MDA-MB-468 cancer cell lines were cultured in 175 cm² sterile cell culture flasks (CELLSTAR® Greiner Bio-One GmbH Kremsmünster, Austria) containing 25 to 30 mL of the culture medium. A431 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco® by Life Technologies™, Paisley, UK) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (PS). For maintaining MDA-MB-468 cells, DMEM:Ham’s F-12 1:1 mix (Lonza BioWhittaker® Verviers, Belgium) containing 10% FBS and 1% PS was used. To assure cell growth, cells were kept at 37°C and 5% CO₂ in a humidified atmosphere incubator (SANYO electric co., Ltd, Moriguchi, Osaka) and routinely tested for bacterial or yeast infection with a microscope (Carl Zeiss B.V. Sliedrecht, The Netherlands). When cells reached 70-80% confluency, they were passed according to the preferred dilution to keep the cells in culture.

Culture medium was removed followed by a washing step with 10 mL Dulbecco’s phosphate buffered saline (PBS) (Sigma-Aldrich Co., St. Louis USA). Next, 3 mL 1x Trypsin-Ethylendiaminetetraacetic acid (T/E) was used to detach the cells and the flask was placed in the incubator at 37°C to ensure the optimal operating temperature of the enzyme. When all cells were detached, T/E was inhibited by pipetting at least 9 mL 10% FCS containing DMEM medium. A volume of the total volume in the flask was then transferred into new flasks according to the desired dilution and filled up with DMEM to 25 mL. A431 cells were passed routinely at 1:10 – 1:12 dilution whereas MDA-MB-468 cells were passed at 1:4 - 1:6 dilution. These proceedings were always performed in a
dedicated biosafety lab (ML-1) in a lamellar flow cabinet and the operating person wore the proper gear including a lab coat and gloves.

3.2 ASSAY PREPARATION

To obtain EV-s, cell culture medium was replaced with fresh Opti-MEM® I + GlutaMAX™ Reduced Serum Medium (Gibco® by Life Technologies™, Paisley, UK) for 48 hours. This medium is reduced in serum and stresses the cells which leads to a higher grade of vesiculation\textsuperscript{87}. After this incubation period, Opti-MEM® medium was removed from the cells, followed by centrifugation with a Rotanta 96 R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) at 300 G for 10 minutes and 2000 G for 10 minutes to remove remaining cells and cell debris.

3.3 SIZE AND CONCENTRATION OF EVs

Determination of the size distribution and concentration of EVs in the centrifuged medium was achieved using nanoparticle tracking analysis (NTA) using a NanoSight LM14 C instrument (Malvern Instruments Company, Malvern, UK) and NTA software 2.3.

Samples were diluted in PBS prior to measurements. In general, a 10 fold dilution was used for appropriate measurements. All the tubing and optics were rinsed with water, cleaned with 70\% ethanol and dried with a combination of compressed air and lens cleaning tissues (Laméris Ootech BV, Nieuwegein, The Netherlands).

The sample was introduced by a 1 mL syringe using the inlet port of the NanoSight module and slowly injecting approximately 500 \(\mu\)L sample which was sufficient to fill the measuring module. A blue laser with a wavelength of 488 nm was used for scattering of particles. The temperature of the laser was continuously kept on 22\(^\circ\)C during measurements. Each sample was measured 5 times through subsequently injecting a fraction of 100 \(\mu\)L for 60 seconds while a video was recorded. Per sample, a batch analysis was performed on the five recorded videos. NTA software calculated size, size distribution and concentration depending on the Brownian motion of the vesicles. A batch analysis required fine-tuning of the analyzing parameters such as detection threshold, camera level, screen gain and minimum expected particle size as they can
have an influence on the given size and concentration. The value of these parameters was up to the user to decide depending on the kind of sample. These parameters were kept constant during the different measurements of the same sample. In between different samples the NanoSight module, optic and tubing were cleaned thoroughly as stated above.

3.4 IMMUNOCAPTURE BEAD ASSAY

Characterizing surface proteins of EVs was achieved using a magnetic immunocapture bead assay. Cell medium was collected and prepared for the assay as described under 3.2. ExoCap™ kits CD9, CD81, CD63, EpCAM or Composite (mixture of tetraspanins CD9, CD81 and CD63) (JSR Life Sciences Micro®, Leuven, Belgium) were used and diluted to 10000 beads/µL with PBS. These beads are coated with a JSR Life Sciences® proprietary polymer and cofactors to prevent coupling between beads. 5µL of beads solution was added in every well of a U-bottom 96-well plate (Thermo Fisher Scientific™ Nunc™ A/S Roskilde, Denmark). The bead solution was incubated with 200 µL prepared cell medium overnight at 4°C on a Titramax 100 shake plate at 600 rpm (Heldolph Instruments, Schwabach, Germany). The medium was removed the next day using a magnetic plate to keep the magnetic EV-bead complexes at the bottom of the well plate. The plate was washed using 150 mL PBS containing 2% bovine serum albumin (BSA) and the plate was placed on the shaker plate for 5 minutes at 600 rpm. PBS containing 2% BSA was removed using the magnetic plate and the wells were incubated with 100 µL of diluted Alexa Fluor® 647 mouse anti-human antibodies (e.g. CD9, CD81, CD63, CD147, EpCAM, EGFR, HER2 etc) (BD Pharmingen™, San Diego, USA) in PBS. As a control, one row of wells was not incubated with antibodies and one row was incubated with isotype control antibody. Incubation was performed for 2h at room temperature (RT) on the shake plate at 600 rpm. After the incubation, the plate was washed with 2% BSA in PBS as described before. Prior to analyzing the plate with flow cytometry, 150 µL 0,25% BSA in PBS was added to the wells (Figure 3.1). Beads only (BO) and isotype control antibody (ISO) were used to assess the level of background. The BO signal gives the intrinsic background signal of the immunocapture beads incubated with medium. Isotype control antibody has no specificity for human tetraspanins or tumor makers yet it retains all the non-specific properties of the antibodies used.
Figure 3.1: An overview of the immunocapture bead assay method and NTA for the characterization of EVs. Cell culture medium was centrifuged (10 min. 300 G, 10 min. 2000 G). Size distribution and concentration of EVs was determined using NTA. EVs were captured and enriched using immunobeads followed by incubation with fluorescent antibodies and mean fluorescence intensity was determined using flow cytometry.

3.5 STREPTAVIDIN EGFR IMMUNOCAPTURE BEAD ASSAY

JSR Life Sciences Micro® supply CD9, CD63, CD81, composite and EpCAM capture kits. To enrich vesicles that expose EGFR on their membrane, streptavidin – EGFR conjugated beads were made. Invitrogen™ Dynabeads® M-280 Streptavidin (Life Technologies™, Paisley, UK) were prepared to conjugate with EGFR biotin antibodies (Abcam®, Cambridge, UK). Four µL, roughly 2.6 x 10⁶ beads, from a stock solution of ~6-7 x 10⁸ beads/mL was transferred into an Eppendorf®. First, the beads were diluted with 1 mL PBS and the solution was vortexed. The solution was placed on a magnetic plate until the magnetic beads formed a pellet and the supernatant was discarded.

The beads were then resuspended in 1 mL PBS and incubated overnight at 4°C with 8 µL of biotinylated EGFR antibodies on a shaker plate. The next day, the bead solution was washed four times as described above. The beads were resuspended in 260 µL to become a solution of ~10000 beads/µL. Similar to the immunocapture bead assay, 5 µL/well was added which equals 50000 beads/well.
3.6 CELL ASSAY

To investigate the relation between the expression of surface proteins on the tumor cells and their corresponding vesicles, flow cytometry experiments with the tumor cells were performed. Opti-MEM® cell medium was removed and the cells were washed with PBS at RT. After the washing step, cells were detached with T/E. The enzyme was inhibited and cells were resuspended using at least 9 mL of the cell culture medium. The cell suspension was transferred into a 50 mL tube and centrifuged at 300 G for five minutes. Ten mL fresh cell medium was used to resuspend the cell pellet. Twenty µL trypan blue (Sigma-Aldrich Co., St. Louis USA) was added to 20 µL cell suspension and the cells were counted using a Bürker-Türk hemocytometer (Marienfeld Superior, Lauda-Königshofen, Germany). After counting, the cell suspension was centrifuged at 300 G for five minutes and the obtained pellet was diluted in cold PBS at 4°C to reach a concentration of 500000 cells/mL.

Hundred µL of the cell suspension (50000 cells) was pipetted in each well of the 96-well plate. The plate was centrifuged at 500 G for 5 minutes at 4°C. The supernatant was removed and the cells were incubated for 2 hours with in PBS containing 0.25% BSA-diluted Alexa Fluor® 647 mouse anti-human antibodies. The well plate was protected from light during the incubation period. The plate was centrifuged at 500 G for 5 minutes at 4°C and 150 µL PBS containing 0.25% BSA was used to resuspend the pellet. The well plate was again centrifuged using the same parameters and the pellet was resuspended with 150 µL PBS 10% formalin to fix the cells. Similar to immunocapture beads assay, cells only (CO) and isotype control antibody (ISO) were used to assess the level of background signal.

3.7 5-ALA ASSAY

3.7.1 5-ALA concentration

A 10 mg/mL solution of 5-ALA (Alfa Aesar®, Massachusetts, USA) was prepared using PBS. This was equal to a concentration of 60 mM with 167,59 Da being the atomic mass of 5-ALA. 833 µL of this solution was diluted to 50mL using Opti-MEM® cell medium reaching a concentration of 1mM 5-ALA in the cell medium. Twentyfive
23 mL of this solution was used to incubate the cells for 2 days at 37°C and 5% CO₂ in an incubator.

3.7.2. Preparation for PpIX detection in EVs

Harvesting and adding the beads was performed according to the method described in section 3.2 and 3.4 except no fluorescent antibodies were added in this assay. When the medium was removed, it was directly pipetted into a black 96-well plate (Thermo Fisher Scientific Nunc® A/S Roskil, Denmark). The beads in the normal well plate were analyzed with flow cytometry. The black well plate was analyzed using a Spectramax M2 fluorimeter (Molecular Devices, Sunnyvale, USA). The excitation wavelength was set on 405 nm and the emission wavelength was set on 635 nm (Figure 3.2).

![Figure 3.2: The absorption spectra and normalized emission spectra of PpIX. Average μₐ and μ₂ absorption spectra of ex vivo rat brain tissue, measured at the cerebral cortex. Excitation maximum at 405 nm and emission maximum at around 635 nm. Kim et al.89](image)

3.7.3. Preparation for PpIX detection in cells

Cells are prepared as described in section 3.5 except no fluorescent antibodies were added due to the intrinsic fluorescence of PpIX.
3.8 FLOW CYTOMETRY

A BD FACSCanto™ II Flow Cytometer (BD Biosciences, San Jose, USA) was used to analyze cells and the cell-derived EVs. This flow cytometer was equipped with a blue laser (488 nm) and a red laser (633 nm) and a BD Biosciences High Throughput Sampler to process 96-well plates.

3.8.1. Immunocapture bead and streptavidin – EGFR bead assays

Before running the complete well plate, a single well of beads only was processed to set a correct gate for the SSC and the FSC of the beads in combination with the blue laser (488 nm). The allophycocyanin channel (APC) was used with 660/20 BP in combination with the red laser with a wavelength of 633 nm. These beads are not expected to have a high mean APC signal due to the absence of the Alexa Fluor 647® antibodies. The following parameters for the bead assay were used:

- FSC: 400 V
- SSC: 225 V
- APC: 545 V
- Mixing speed: 200 µL/sec
- Wash: 800 µL
- Flow rate: 2,5 µL/sec
- Volume: 100 µL
- Mixing: 100 µL
- Number of mixes: 5

3.8.2. Cell assay

Parameters were the same as described in section 3.8.1., but other parameters for FSC, SSC and APC were used:

- FSC: 180 V
- SSC: 250 V
- APC: 500 V

3.8.3. 5-ALA assay

5-ALA is converted into PpIX which has fluorescent capacities. PpIX has an excitation peak at 405 nm and an emission maximum at 635 nm (Figure 3.2).
3.8.3.1. PpIX detection in EVs

Parameters were the same as described in section 3.8.1.

3.8.3.2. PpIX detection in cells

FACS parameters were the same as described in section 3.7.1 except that an extra R-phycoerythrin (PE) channel was added with voltage of 560 and with 610/20 BP in combination with an extra blue laser with a wavelength of 488 nm.

3.8.4. CytoFLEX

A new generation of flow cytometer was used called a CytoFLEX® (Beckman Coulter, California, USA). This flow cytometer is equipped with 3 lasers and 13 fluorescence channels affording the capabilities to adapt to changing experimental conditions. Besides the red and blue laser, the CytoFLEX® is also equipped with a violet laser (405 nm) which enables the apparatus to detect nanoparticles. Cell medium preparation was performed as described in section 3.4, but only CD9 immunocapture beads and no detection antibodies were used. For CytoFLEX® measurements, the violet laser of 405 nm was used in combination with a detector at 635 nm. This allowed to measure in the maximum absorption and emission peaks of PpIX. Emission was measured using detection at 638 nm and the filter was called ‘Filter PpIX’. Violet detectors at 610 and 660 nm were used as a control. Following voltages were:

FSC: 80 V
SSC: 171 V
Filter Violet 610 nm: 99 V
Filter Violet 660 nm: 147 V
Filter PpIX 638 nm: 4 V

3.9 ELISA

A white flat bottom 96-well plate (Thermo Fisher Scientific™ Nunc™ A/S Roskil, Denmark) was coated with 4 µg/mL EGFR antibody (Abcam®, Cambridge, UK). The antibody was diluted in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 3 mM NaN₃) and 55 µL/well was added. Incubation occurred overnight at 4°C during static conditions.
The next day, the excess antibody coating solution was removed by tapping and washing the plate three times using 150 µL/well 0.05% Tween 20 in PBS. One Hundred fifty µL/well blocking buffer (1% BSA in PBS) was added to the plate and incubated on a shaker at 600 rpm for one hour at RT. The blocking buffer was removed by tapping the plate and the plate were washed. Cell culture medium was added to the wells ranging from 25 to 200 µL/well. Volumes less than 200 µL were filled up with pure Opti-MEM® to 200 µL. The medium was removed by tapping and washing the plate three times. The EGFR coated plate was incubated with 100 µL/well detection biotin antibody EGFR (Abcam®, Cambridge, UK). Detection antibody was diluted with PBS 1% BSA to reach a concentration of 1 µg/mL. Incubation occurred on a shaker at 600 rpm for 1 hour at RT. The antibody solution was removed by tapping and washing three times. One hundred µL/well Streptavidin-multi horseradish protease (HRP) (1.0 mg/ml Sanquin, Amsterdam, The Netherlands) diluted 1:5000 in PBS 1% BSA was added to each well of the plate and incubated on a shaker at 600 rpm for 30 minutes at RT. Plate was tapped and washed three times. SuperSignal® ELISA Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, USA) was prepared right before adding 100 µL to the wells and incubated on a shaker plate at 600 rpm and luminescence was measured after 15 and 30 minutes. The plate was protected from light using aluminum foil. Chemiluminescent signal was detected using a Synergy 2 microplate reader (BioTek®, Winooski, USA). Twenty-four control wells were used to assess the level of background luminescence. Eight were filled with 200 µL Opti-MEM®, in the other eight wells no detection biotinylated EGFR antibody was added and in the last eight wells no HRP was added.

3.10 LYSATES

Cells were lysed using a solution of 990 µL RIPA buffer (Teknova, California, USA) and 10 µL HALT protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific™, Waltham, USA). Medium was removed from the cells and the cells were washed with 10 mL PBS. Cells were detached using 3 mL T/E and resuspended with DMEM 10% FCS. The cell suspension was transferred to a 50 mL tube and centrifuged at 300 G for five minutes. Supernatant was removed and the cells were resuspended in PBS at 4°C. The suspension was centrifuged at 300 G for five minutes but at 4°C. Supernatant was removed and the pellet was resuspended with the 1 mL RIPA buffer HALT cocktail solution. After 20
minutes on ice, the cell suspension was transferred to an Eppendorf® and centrifuged at 14000 G for 15 minutes at 4°C. Supernatant was removed and stored at -20°C. Vesicle lysates were not prepared as they were already prepared in stock.

3.11 SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

A Novex® Bolt™ Mini Gel Tank with a precast Novex® Bolt 4-12% Bis-Tris Plus polyacrylamide gel (Life Technologies™, California, USA) was used to perform SDS-PAGE. SDS was used to ensure the denatured state of the proteins and their negative charge. The precast gel was rinsed 3 times with Bolt™ MOPS SDS running buffer 1X (Invitrogen®, California, USA). The precast gels were placed in both chambers of the mini gel tank and the chambers were filled with 1X MOPS SDS running buffer. Cell and vesicle samples were prepared by adding H2O and/or 4x reducing sample buffer (SB) to the cell and vesicle lysates in a way that there was a concentration of 10 µg lysate in 20 µL sample. Reducing SB consists out of 40% glycerol, 240 mM Tris/HCl pH 6,8, 8% SDS, 0,04% bromophenolblue and 5% beta-mercaptoethanol. 60 µL of each sample was prepared as seen in table 3.1.

Table 3.1: Cell and vesicle sample preparation for SDS-PAGE

<table>
<thead>
<tr>
<th>Sample</th>
<th>Name</th>
<th>[lysate ug/ul]</th>
<th>uL lysate</th>
<th>uL H2O</th>
<th>uL SB (4x)</th>
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<td>42.5</td>
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<td>MDA-MB-468 cells</td>
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<td>10.3</td>
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</tr>
<tr>
<td>3</td>
<td>MCF-7 cells</td>
<td>6.59</td>
<td>4.6</td>
<td>40.4</td>
<td>15.0</td>
</tr>
<tr>
<td>4</td>
<td>SK-BR-3 cells</td>
<td>7.59</td>
<td>4.0</td>
<td>41.0</td>
<td>15.0</td>
</tr>
<tr>
<td>5</td>
<td>A431 EVs</td>
<td>/a</td>
<td>45.0</td>
<td>0.0</td>
<td>15.0</td>
</tr>
<tr>
<td>6</td>
<td>MDA-MB-468 EVs</td>
<td>/a</td>
<td>45.0</td>
<td>0.0</td>
<td>15.0</td>
</tr>
<tr>
<td>7</td>
<td>MCF-7 EVs</td>
<td>/a</td>
<td>45.0</td>
<td>0.0</td>
<td>15.0</td>
</tr>
<tr>
<td>8</td>
<td>SK-BR-3 EVs</td>
<td>/a</td>
<td>45.0</td>
<td>0.0</td>
<td>15.0</td>
</tr>
</tbody>
</table>

*aDue to an unknown lysate concentration from cell line EVs, no dilution is made with H2O.

MCF-7 and SK-BR-3 are cell lines that were taken along as a control. SK-BR-3 cell line has a high expression of HER2 and a much lower expression of EGFR compared to A431 and MDA-MB-468. MCF-7 cell line has nor a high expression of EGFR nor a high expression of HER2. Both MCF-7 and SK-BR-3 are breast adenocarcinoma cell lines. Samples were
then heated at 95°C for 5 minutes and 20 µL of each sample was loaded into wells accompanied by 5µL of the Precision Plus Protein™ Standards (BIORAD laboratories, California, USA). The SDS-PAGE was performed for 45 minutes at 400 mA and 165 V.

3.12 WESTERN BLOTTING

The blotting process was performed by making a sandwich of the polyacrylamide gel of the SDS-PAGE with an Immobilon®-FL polyvinylidene fluoride (PVDF) transfer membrane (Merck Millipore Ltd., Tullagreen Carrigtwohill, Ireland). Before blotting, attention was paid to keep this membrane under methanol. A BIORAD cassette (BIORAD laboratories, California, USA) was prepared for blotting in following order; a Pre-wetted fiber pad, two filter papers (Whatman, Little Chalfont, UK), the activated PVDF membrane, the polyacrylamide gel and again two filter papers and a pre-wetted fiber pad. When no air bubbles were trapped, the cassette was closed and locked down in place in a BIORAD blotting container accompanied by a Bio-Ice cooling unit to keep the heat down (BIORAD laboratories, California, USA). This container was filled with blotting buffer (6.06 g Tris, 28.8 g Glycine dissolved in 1600 mL aqua dest. and 400 mL ethanol). The electrodes were coupled to the unit and the blotting was performed for one hour at 125 V.

Next, each blot was cut into pieces containing the proteins of interest and rinsed with aqua dest. Blocking buffer was prepared by making a 1:1 dilution of Odyssey® Blocking Buffer (LI-COR®, Nebraska, USA) and a 1/10 diluted 10x Tris-buffered saline (TBS) solution. 10 mL of this solution was added to each blot in a 50 mL tube. Each blot was in a separate 50 mL tube and incubated for two hours on a roller bench at RT. The antibody incubation buffer Odyssey® was prepared by diluting Odessey® blocking buffer 1:1 with TBS-Tween 20. The primary antibody was added to the antibody incubation buffer in the proper dilution (1:1000). The blocking buffer was discarded and the antibody incubation buffer with the primary antibody was added to the blots in the 50 mL tubes (Figure 3.3). The blots were again incubated for two hours on a roller bench at RT. Loading control antibodies are needed to control whether the same amount of sample was loaded into each well during SDS-PAGE. Also it gives an insight in the efficiency of the transfer of proteins from the gel onto the membrane and provides
uniformity of signal detection of samples. β-actin which has a molecular weight of 45 kD was used as a loading control for cell lysates. It is a protein secreted by eukaryotic cell types in large quantities. However, it is not an appropriate EV marker. CD9 instead was used as a potential EV marker.

Figure 3.3: Western Blot on cell and vesicle lysates of A431, MDA-MB-468, MCF7 and SK-BR-3 cell lines. Incubation of the blot with primary antibodies: rabbit EGFR antibody (Cell Signaling Technologies, Danvers, USA), mouse β-actin antibody (Cell Signaling Technologies, Danvers, USA) and rabbit anti CD9 antibody (Abcam®, Cambridge, UK). The blot was cut into three pieces. After the incubation, the blots were washed three times with Tween 20-TBS and once with TBS solution. New antibody incubation buffer was prepared and the secondary antibodies against the primary antibodies were added in the proper dilution (1:2500). The blots were incubated for 2 hours on a roller bench at RT and protected from light and afterwards washed three times with Tween 20-TBS and once with TBS solution. The blots were scanned using an Odyssey® infrared imager (LI-COR®, Nebraska, USA). Sensitivity settings and other parameters were up to the operator and depended on the blot.
4. RESULTS

4.1 IMMUNOCAPTURE BEAD ASSAYS

4.1.1. Tetraspanin detection on A431 cells and A431-derived EVs

Different subpopulations of tumor cell-derived EVs display tetraspanins e.g. CD9, CD81 and CD63 on their surface. Immunobeads were used to enrich these specific subpopulations followed by an incubation of these bead-coupled EVs with fluorescent detection antibodies against these tetraspanins. This method is able to enlighten different characteristics between subpopulations of EVs secreted by various tumor cell lines (Figure 4.1).

![Tetraspanin detection on A431-derived EVs and A431 cells](image)

Figure 4.1: Tetraspanin detection on A431-cells and EVs. A. A431 tumor cells were cultured in Opti-MEM® for two days. 200 µL medium was incubated overnight at 4°C with ExoCap™ immunocapture beads. After the incubation, anti-human fluorescent antibodies against the tetraspanins CD9, CD63, CD81 were added and the mean fluorescence intensity (MFI) was determined by flow cytometry. Beads only (BO) and Isotype control antibody (ISO) were used to assess the level of
background. Data are presented as mean ± SD of one representative experiment performed in triplicate. B. A431 cell suspension was incubated with fluorescent antibodies for 2 hours at 4°C. Cells only (CO) and Isotype control antibody (ISO) were used to assess the level of background. Data are presented as mean ± SD of one representative experiment performed in triplicate.

High CD9 and CD81 detection in comparison to CD63 detection was observed on EVs captured by each type of ExoCap™ immunocapture beads, especially for CD9 beads. EVs captured by ExoCap™ CD63 however, gives the lowest signal of all immunocapture beads. Correlation is seen between EVs captured by ExoCap™ CD9, CD81, Composite and tetraspanin detection on A431 cells, suggesting that A431-derived EVs mirror the tetraspanin expression of their parent cells.

4.1.2. Tumor marker detection on A431 cells and -derived EVs

Many tumor cells display distinct proteins on their surfaces. These proteins, also known as tumor markers, can be displayed on the surfaces of tumor cell-derived EVs. The expression of tumor markers on the cell seems to correlate with the detection of these proteins on the surface of the derived EVs (Figure 4.2).

Overall, the obtained signals using anti-tumor marker antibodies are relatively low. Enrichment with ExoCap™ CD9 immunobeads gave the highest MFI. Correlation is seen between EVs captured by ExoCap® CD9 immunobeads and tumor marker expression on A431 cells, affirming the possibility of tumor cell-derived EVs to mirror the tumor marker expression of their parent A431 cells.
Figure 4.2: Tumor marker detection on A431 cells and EVs. A. A431 tumor cells were cultured in Opti-MEM® for two days. 200 µL medium was incubated overnight at 4°C with ExoCap™ immunocapture beads. After the incubation, anti-human anti-tumor marker fluorescent antibodies were added and the MFI was determined by flow cytometry. Data are presented as mean ± SD of one representative experiment performed in duplicate. No HER-2/CD147 data shown for ExoCap™ EpCAM and Composite. B. A431 cells were incubated with fluorescent antibodies for 2 hours at 4°C. Data are presented as mean ± SD of one representative experiment performed in triplicate. CO and ISO were used to assess the level of background.

4.1.3. Tetraspanin detection on MDA-MB-468 cells and -derived EVs

High CD9 and CD81 detection is seen on EVs captured by ExoCap™ CD9 and CD81 immunocapture beads, especially for CD9 beads. ExoCap™ CD63 however, gives the lowest signal of all immunocapture beads. Figure 4.3 shows correlation between EVs captured by ExoCap™ CD9, CD81 and tetraspanin detection on MDA-MB-468
cells suggesting that MDA-MB-468-derived EVs mirror the tetraspanin expression of their parent cells.

Figure 4.3: Tetraspanin detection on MDA-MB-468-derived EVs. A. A431 tumor cells were cultured in Opti-MEM® for two days. 200 µL medium was incubated overnight at 4°C with ExoCap™ immunocapture beads. After the incubation, anti-human fluorescent antibodies against the tetraspanins CD9, CD63, CD81 were added and the MFI was determined by flow cytometry. Beads only (BO) and Isotype control antibody (ISO) was used to assess the level of background. Data are presented as mean ± SD of one representative experiment performed in triplicate. B. MDA-MB-468 cells were incubated with fluorescent antibodies for 2 hours at 4°C. Data are presented as mean ± SD of one representative experiment performed in triplicate.
4.1.4. Tumor marker detection on MDA-MB-468 cells and -derived EVs

Figure 4.4 shows correlation between EVs captured by ExoCap™ CD9, CD63, CD81 and tumor marker detection on MDA-MB-468 cells suggesting that MDA-MB-468-derived EVs mirror the tumor marker expression of their parent cells.

Figure 4.4: Tumor marker detection on MDA-MB-468 cells and EVs. A. MDA-MB-468 tumor cells were cultured in Opti-MEM® for two days. 200 µL medium was incubated overnight at 4°C with ExoCap™ immunocapture beads. After the incubation, anti-human anti-tumor marker fluorescent antibodies were added and the MFI was determined by flow cytometry. Data are presented as mean ± SD of one representative experiment performed in duplicate. B. MDA-MB-468 cells were incubated with fluorescent antibodies for 2 hours at 4°C. Data are presented as mean ± SD of one representative experiment performed in triplicate.

4.1.5. Different filtration degrees of cell culture medium

The effect of different sized filters for processing Opti-MEM® for the detection of A431-derived EVs (Figure 4.5) was investigated. CD9 and CD81 ExoCap™
immunobeads were used to capture the EVs. Medium was filtered using a syringe and a membrane filter with a diameter of 0,22 µm (Corning™, New York, USA) or 0,45 µm (Sartorius™, Göttingen, Germany). NTA data shows no significant difference both in size distribution between the different filtration degrees. As for the total concentration of EVs, differences were observed between the various conditions (Table 4.1). This had no influence on the immunocapture bead assay because the MFI is approximately the same between the different conditions (Figure 4.5).

![Tetraspanin detection on filtered A431-derived EVs](image)

Figure 4.5: Tetraspanin detection on filtered A431-derived EVs. Tumor cells were cultured in Opti-MEM® for two days. 200µL of Opti-MEM® was incubated overnight at 4°C with ExoCap™ immunocapture beads CD9 and CD81. Anti-human, anti-tetraspanin detection antibodies were added and the MFI was determined by flow cytometry. Data are presented as mean ± SD of one representative experiment performed in quadruplicate.

<table>
<thead>
<tr>
<th></th>
<th>Mean Size (SD)</th>
<th>Particles/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>No filter</td>
<td>140 ± 5,1 nm (70 ± 1,7 nm)</td>
<td>4,74 ± 0,83 E9</td>
</tr>
<tr>
<td>0,2 µm filter</td>
<td>136 ± 3,0 nm (65 ± 7,5 nm)</td>
<td>3,66 ± 0,30 E9</td>
</tr>
</tbody>
</table>
Opti-MEM® medium samples from the different filtration conditions were ten times diluted.

Mean size and total concentration expressed as particles/mL were determined using NTA. Camera level was set at 12 and the detection threshold was set at 10.

4.1.6. Different storage options of EVs in Opti-MEM® medium

If storage of EVs is possible without influence on the detection, assays do not have to be performed immediately after harvesting. Opti-MEM® medium was harvested from cells and stored in three different ways; 5 times freeze thaw cycles on one day, 4 days at -4°C, and 4 days at -20°C (figure 4.6).

4.1.6.1. Storage of A431 EVs

As seen in figure 4.6, assays performed with fresh medium or after five times freeze thaw cycles do not differ much except on EGFR detection. For four days at -20°C however, a decrease in obtained signal is seen for every tetraspanin and for EGFR. Moreover, EGFR and CD63 were not even detected. For four days at 4°C, an increased signal is observed. Important to notice is that in general, the results for the different storage options are in the same order as the ones obtained using fresh medium.

![Figure 4.6: Effect of storage on the detection of tetraspanins and EGFR on A431-](image-url)
derived EVs. Tumor cells were cultured in Opti-MEM® for two days. 200µL of Opti-MEM® was incubated overnight at 4°C with ExoCap™ CD9. The mean of BO and ISO was subtracted from the results. Data are presented as mean ± SD of one representative experiment performed in quadruplicate.

4.1.6.2. Storage of MDA-MB-468 EVs

The results for MDA-MB-468 cells (Figure 4.7) are similar to the results for A431 cells seen in Figure 4.6. The signals obtained with different storages methods are in the same order as the ones obtained using fresh medium.

![Detection of tetraspanins and EGFR on MDA-MB-468-derived EVs](image)

Figure 4.7: Effect of storage on the detection of tetraspanins and EGFR on MDA-MB-468-derived EVs. MDA-MB-468 cells were cultured in Opti-MEM® for two days. 200µL of Opti-MEM® was incubated overnight at 4°C with ExoCap™ CD9. The mean of BO and ISO was subtracted from the results. Data are presented as mean ± SD of one representative experiment performed in quadruplicate.

4.1.7. EV detection with different fluorescent antibody concentrations

This assay was done to check whether the correct concentration of antibody was used for the detection of potential EV and tumor markers. Linear detection was observed on each type of ExoCap™ immunocapture beads except for EGFR detection (Figure 4.8). In previous assays, used antibody concentrations were 0,5
µL/well for CD9 and CD63 antibodies; 1 µL/well for EGFR antibody and 2 µL/well for CD81 antibody. Figure 4.8 shows that a correct balanced was found between obtained MFI and the amount of used antibodies.

Figure 4.8: EV detection with different concentration of fluorescent antibodies. A431 cells were cultured in Opti-MEM® for three days. 200 µL of Opti-MEM® was incubated overnight at 4°C with ExoCap™ CD9, CD63 and CD81 immunocapture beads. Data are presented as mean ± SD of one representative experiment performed in triplicate.

4.1.8. EV detection in different cell culture media

In all of the experiments, ExoCap™ immunocapture beads were incubated with Opti-MEM® cell culture medium as described in section 3.2. To determine the influence of the medium on measurements, three different media were analyzed; Opti-MEM®, serum-free DMEM and depleted EV medium (DEM) (Figure 4.9). DEM was prepared by centrifuging DMEM 10% FCS. FCS contains serum and serum vesicles which are removed by centrifuging at 100000 G for 16 hours and filtered through a 0.22 µm filter.
Figure 4.9: Tetraspanin and tumor marker detection on A431-derived EVs. A431 cells were cultured in Opti-MEM®, DEM and DMEM for two days. 200 µL of each medium was incubated overnight at 4°C with ExoCap™ CD9. Graph based on results in duplo and experiment was repeated three times. Data are presented as mean ± SD of three representative experiment performed in duplicate.

Although the MFIs of EVs in DEM and DMEM are in the same order, they are lower than the MFI obtained with Opti-MEM® (Figure 4.9). As seen in figure 4.10 which gives the results normalized for Opti-MEM®, MFIs for DEM and DMEM are reduced by half.
Figure 4.10: MFIs normalized for Opti-MEM®. Results for CD9, EGFR and CD147 detection normalized for Opti-MEM®. Data are presented as mean ± SD of one representative experiment performed in triplicate.

As seen in table 4.2, NTA quantification of the total number of particles was the greatest in serum-free DMEM and DEM. The mean size was the greatest for vesicles present in Opti-MEM®, but Opti-MEM® has the lowest concentration of particles/mL. It can be assumed that differences in culture media can affect the quantity and composition of A431-derived EVs as seen in figure 4.9 and table 4.2 together.

Table 4.2: NTA data different cell culture media

<table>
<thead>
<tr>
<th></th>
<th>Mean Size (SD) b</th>
<th>Particles/ mL b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opti-MEM® a</td>
<td>174 ± 2.7 nm (84 ± 3.7 nm)</td>
<td>1.38 ± 0.07 E9</td>
</tr>
<tr>
<td>DMEM a</td>
<td>148 ± 2.8 nm (69 ± 2.6 nm)</td>
<td>3.54 ± 0.18 E9</td>
</tr>
<tr>
<td>DEM a</td>
<td>168 ± 31.1 nm (97 ± 36.6 nm)</td>
<td>2.52 ± 0.32 E9</td>
</tr>
</tbody>
</table>

aOpti-MEM®, DMEM and DEM medium samples ten times diluted in PBS.
bMean size and total concentration expressed as particles/mL were determined using NTA. Camera level was set at 12 and the detection threshold was set at 10.
4.1.9. Streptavidin EGFR immunocapture beads assay

Dynabeads were prepared as described in section 3.5. Using EGFR immunocapture beads, it is possible to capture and analyze different EVs subpopulation in comparison with previous assays done with the ExoCap™ kits.

**Detection of tetraspanins and EGFR on A431-derived EVs**

![Bar chart](image)

**Figure 4.11:** Detection of tetraspanins and EGFR on A431-derived EVs. A431 cells were cultured in Opti-MEM® for three days. 200 µL of medium was incubated with ExoCap™ CD9 kit and with the prepared EGFR beads. The mean of BO and ISO was subtracted from the results. Data are presented as mean ± SD of one representative experiment performed in quadruplicate.

Figure 4.11 shows a slightly higher intensity for EGFR detection on the EVs enriched with the Dynabeads® especially for MDA-MB-468 cells. P1 and P4 stands for different populations as seen on the analysis report of the flow cytometer. It is feasible that the Dynabeads® form doublets and even triplets causing a higher MFI as seen in Figure 4.12. The P1 population represents all of the subpopulations and the P4 population represents single beads only.
Figure 4.12: Analysis report BD FACSCanto™ II Flow Cytometer. P1 represents all of the subpopulations. P4 represents the single beads. P5 and P6 represents respectively the doublets and the triplets formed by the beads.

4.2 5-ALA ASSAYS

Preparation of the assay is described in section 3.7. Experiments were performed to investigate the relation between 5-ALA uptake and conversion to PpIX in the cells and the presence of PpIX in the secreted vesicles.

4.2.1. PpIX detection in A431 cells

A vast MFI is observed from the A431 cells incubated with 1mM 5-ALA medium in comparison with the control group. This suggests that 5-ALA is taken up by the cells and converted into PpIX casuing the fluorescent signal (Figure 4.13). Cells were measured using BD FACSCanto™ II Flow Cytometer with both the red (633 nm) and blue laser (488nm) in combination with detection channels; APC (660/20) and PI (610/20).
Figure 4.13: PpIX detection in A431 cells. One flask of A431 cells were cultured in Opti-MEM® and in the other flask was incubated with Opti-MEM® 1 mM 5-ALA. Both were cultured for two days and measured using BD FACSCanto™ II Flow Cytometer. Data are presented as mean ± SD of one representative experiment performed in sextuplicate.

4.2.2. PpIX detection in A431-derived EVs

EVs were prepared as described in section 3.7.2 and flow cytometer parameters were set as described in section 3.8.3.1. A431 cells were cultured in Opti-MEM® for two days and ExoCap™ CD9 immunocapture beads were used to enrich the EVs. PpIX was excited using the blue laser (488 nm) and signal was detected with the APC channel (660 nm). No MFI was observed, suggesting that maybe no PpIX was transferred from the cells to the EVs or that EVs cannot take up 5-ALA and convert it to PpIX. Results of the fluorimeter however show that the medium is fluorescent, suggesting that there is indeed PpIX present in Opti-MEM® (Figure 4.14). It is likely that there is PpIX present in the EVs due to the fluorescence signal from Opti-MEM®. Absence of fluorescent signal from the EVs can be the cause of not measuring at the maximum absorption and emission wavelengths of PpIX.
Figure 4.14: Fluorescence of Opti-MEM®. A431 cells were cultured for two days on Opti-MEM® and on Opti-MEM® with 5-ALA. The given amount of Opti-MEM was transferred into wells of a black 96-well plate and diluted to 200 µL with pure Opti-MEM®. The excitation wavelength was set on 405 nm and the emission wavelength was set on 635 nm. Data are presented as mean ± SD of one representative experiment performed in quintuplicate.

Therefore this experiment was repeated using the CytoFLEX. With this apparatus excitation is possible at 405 nm using the violet laser and PpIX emission can be detected at 638 nm close to its maximum emission wavelength (Figure 3.2 in section 3.7.2.). Settings for the CytoFLEX are described in section 3.8.4. Again, no MFI was observed using the CytoFLEX. This suggests that there is no PpIX present in the EVs nor do they take up 5-ALA and covert it to PpIX.

Table 4.3: NTA data PpIX detection in A431-derived EVs

<table>
<thead>
<tr>
<th></th>
<th>Mean Size (SD)</th>
<th>Particles/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opti-MEM®a</td>
<td>173 ± 5.1 nm (71 ± 7.5 nm)</td>
<td>2.19 ± 0.20 E9</td>
</tr>
<tr>
<td>Opti-MEM with 5-ALAa</td>
<td>139 ± 9.8 nm (75 ± 9.9 nm)</td>
<td>2.44 ± 0.11 E9</td>
</tr>
</tbody>
</table>

aSamples were ten times diluted in PBS.
bMean size and total concentration expressed as particles/mL were determined using NTA. Camera level was set at 12 and the detection threshold was set at 11.
NTA quantification of particle size and total number of particles showed no major differences between the two samples as seen in Table 4.3.

4.3 ELISA

An ELISA was performed to give more insight in other subpopulations of EVs rather than the subpopulations captured by the ExoCap™ kits. The assay was performed as described in section 3.9. As seen in figure 4.15, Both MDA-MB-468 and A431–derived EVs express the tumor marker EGFR. A431 EVs showed even some linearity.

![ELISA EGFR coating/EGFR detection](image)

**Figure 4.15: ELISA on A431 and MDA-MB-468 EVs.** Cells were cultured on Opti-MEM® for two days. A 96-well plate was coated with EGFR antibody and biotinylated EGFR detection antibody was added. The given amount of culture Opti-MEM was transferred into wells of a 96-well plate and diluted to 200 µL with pure Opti-MEM®. The plate was measured 15 minutes after incubation with substrate. The mean of the control wells was subtracted from the results. Data are presented as mean ± SEM of one representative experiment performed in quadruplicate.

4.4 WESTERN BLOTTING

Western Blotting was performed to visualize and gain further information about the relationship between cells and their EVs. The Western Blot was performed as described in section 3.11 and 3.12. As seen in figure 4.16, every cell line expressed EGFR at 170 kD. The highest signal was observed for A431 cell lysate followed by MDA-MB-468. As
predicted (Table 3.1, section 3.11), the expression of EGFR by MCF7 and SK-BR-3 was lower. The same is observed for the vesicle lysates, although there was a smeared signal for the A431 EVs due to an unknown vesicle lysate concentration and excessive loading. CD9 (25 kD) was present on cell lysates from every cell line but only present on A431 and MDA-MB-468 derived EVs. B-Actin was expressed by both vesicle and cell lysates except for SK-BR-3 EVs. Expression of EGFR between cells and their vesicles correlates, this was also expected for CD9 as seen on previous immunocapture bead assays (section 4.1.). MCF7 and SK-BR-3 cell express CD9 on the cells but not on their EVs.

Figure 4.16: Western blot of cell and EVs cell lysates. Detection for β-actin lit up blue due to the fact that the intensity settings were set to high.
5. DISCUSSION

Although immunocapture bead assays are straightforward to perform, variable results were observed. This method, however, is a semi-quantitative method which results in a mean fluorescence intensity of the EV coupled beads, which correlates with the amount of detected surface marker on the vesicles. It does not represent an absolute number of vesicles with the specific surface marker nor can it discriminate between different EV subpopulations. Results can also vary as a result of different Opti-MEM® incubation times and different confluency between cells in culture.

As seen in figure 4.4 and 4.8, tumor marker detection on EVs remains fairly low in comparison with tetraspanin detection, especially for EGFR. With the possible diagnostics and therapeutic capacities of tumor-derived EVs in the near future, detection of EGFR is an important goal. A lot of time was spend on detection of EGFR on EVs captured with JSR micro® ExoCap™ kits coated with tetraspannins. When using the prepared EGFR coated Dynabeads® a higher MFI was observed for EGFR, especially for MDA-MB-468 cells. This can be related to the ExoCap kits capturing a subpopulation of EVs which displays a high amount of CD9 and a low amount of EGFR and the opposite is observed for the EVs captured with the EGFR beads. However, these Dynabeads® are not optimized for these types of assays. In comparison with the JSR micro® ExoCap™ kits which are coated with JSR Life Sciences® proprietary polymer and cofactors, the Dynabeads® do not have a coating besides the streptavidin coating. Due to this uncoated surface, it is likely that beads bound together to form doublets and triplets as seen in figure 4.12. This can also be the reason for the high baseline MFI of 400, produced by the Dynabeads®. When repeating this experiment, Dynabeads® should be coated to decrease non-specific binding and achieve more accurate results.

One of the most promising diagnostic techniques using EVs described in this paper is the 5-ALA assay. If it is possible to detect the fluorescence of PpIX in the EVs, no detection antibodies would have to be used which would save time and money. However, no fluorescence of PpIX in the EVs was observed as described in section 4.2.2. This is contradictory to the results displayed in figure 4.14, which shows a clear and linear fluorescence from Opti-MEM® with 5-ALA. Probably when using ExoCap™ CD9
immunocapture beads, different population of EVs is captured than the population which has PpIX. When repeating this experiment, various types of beads should be used to capture the EV subpopulation of interest that has PpIX within or taken up 5-ALA and converted it to PpIX.

EVs are present in heterogeneous mixtures what makes it difficult for accurate quantification of total concentration and mean size using NTA. Optimization of camera and analysis parameters are up to the operator and thereby subjective. It is likely that the presence of proteins and other non-vesicle impurities which have a similar Brownian movement to EVs are not distinguished leading to an aberrant estimation of size and total concentration.

As discussed by Witwer et al.\textsuperscript{13}, standardization of procedures remains a difficult task. Due to many used methodologies for the analysis and detection of EVs\textsuperscript{12,71–73} standardization is of great importance to provide evidence-based framework for EV detection and analysis. As a result, the observation of fluctuating results between different bead assays and difficulties to compare different bead assays with each other. Proceedings such as incubation times, washing and centrifugation steps, confluence of the cells, etc. can all influence the detection, expression and analysis of EV surface markers due to a yet incomplete understanding of EV subtypes, biogenesis and cargo.
6. CONCLUSION

In this paper, several key features of EV immunodetection were investigated. The main focus of this paper was on the relation of surface marker expression between cells and their vesicles in order to use EVs as a future diagnostics platform. Correlation of expression of EV and tumor makers between cells and their EVs was observed for A431 and for MDA-MB-468 tumor cell lines using immunobead-assisted flow cytometry, Western Blotting and ELISA. Although the MFIs obtained for tumor markers were relatively low in comparison with the tetraspanin detection, there is room for improvement. Higher MFIs could possibly be obtained via development and usage of immunocapture beads with different antibody coatings, e.g. EGFR or CD147 antibody coating to capture other specific EV populations. It was also demonstrated that differences in culture media can affect the quantity and composition of EVs. For the A431 cell line, higher MFIs were obtained from cultures incubated with Opti-MEM® than those with DMEM or DEM. Different filtration degrees however, did not affect EV quantity and composition of A431-derived EVs. Whether or not EV samples can be stored over longer periods of time remains uncertain. The obtained MFIs for EV and tumor markers remain in proportion to each other for the different storage methods, but as observed these intensities can vary according to the different storage circumstances. Thus, immunocapture bead assays are preferably performed with fresh EV samples.

5-ALA was used as a new detection method, making the use of fluorescent antibodies redundant. A431 cells clearly take up 5-ALA and convert it to PpIX causing the high MFI at 635 nm. No fluorescence was seen within the EVs, nor with a conventional flow cytometer, nor with CytoFLEX® at the exact absorption and emission wavelengths of PpIX. Fluorimeter results however, showed linear fluorescence of the EV sample in comparison to the control. Latter suggests that PpIX is present in the EV samples but probably no MFI is measured due to capturing the wrong EV subpopulation. EVs that do have PpIX or picked up 5-ALA and converted it to PpIX are not captured by the immunocapture beads.

In conclusion, analysis of the correlation between EVs and their parent cells concerning the determination of the expression of various tumor markers offers a promising platform for the development of potential future diagnostics tools for cancer and cancer treatment.
7. LITERATURE LIST


Websites and figures:


e. [https://www.beckmancoulter.com/wsportal/wsportal.portal?npfb=true&windowLabel=UCM_RENDERER&urlType=render&wlpUCM_RENDERER_path%3D%252Fwsr%252Fresearch-and-discovery%252Fproducts-and-services%252Fflow-cytometry%252Fflow-cytometers%252Fcytoflex%252Findex.html%23%2F%20%252Fwsr%252Fresearch-and-discovery%252Fproducts-and-services%252Fflow-cytometry%252Fcytometers%252Fcytoflex%252Findex.html](https://www.beckmancoulter.com/wsportal/wsportal.portal?npfb=true&windowLabel=UCM_RENDERER&urlType=render&wlpUCM_RENDERER_path%3D%252Fwsr%252Fresearch-and-discovery%252Fproducts-and-services%252Fflow-cytometry%252Fflow-cytometers%252Fcytoflex%252Findex.html%23%2F%20%252Fwsr%252Fresearch-and-discovery%252Fproducts-and-services%252Fflow-cytometry%252Fcytometers%252Fcytoflex%252Findex.html) (15-05-2015)