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Karolinska Institutet, Stockholm, Sweden

# PURIFICATION, PROFILING AND BIOENGINEERING OF EXTRACELLULAR VESICLES

Giulia Corso


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# Purification, Profiling and Bioengineering of Extracellular Vesicles

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

**Giulia Corso**

*Principal Supervisor:*
Associate Professor Samir EL Andaloussi
Karolinska Institutet
Department of Laboratory Medicine
Clinical Research Center

*Co-supervisor(s):*
Professor C.I. Edvard Smith
Karolinska Institutet
Department of Laboratory Medicine
Clinical Research Center

Imre Mäger, PhD
University of Oxford
Department of Paediatrics
Medical Sciences

Professor Susanne Gabrielsson
Karolinska Institutet
Department of Medicine
Immunology and Allergy

*Opponent:*
Professor Dave Carter
Oxford Brookes University
Department of Biological and Medical Sciences
Health and Life Sciences

*Examination Board:*
Associate Professor Jorge Ruas
Karolinska Institutet
Department of Physiology and Pharmacology
Molecular and Cellular Exercise Physiology

Associate Professor Hadi Valadi
University of Gothenburg
Department of Rheumatology and Inflammation Research
Sahlgrenska Academy

Professor Moustapha Hassan
Karolinska Institutet
Department of Laboratory Medicine
Clinical Research Center

*Alla mia famiglia*

# ABSTRACT

The nano-sized membrane enclosed extracellular vesicles (EVs) transfer macromolecular information in the form of proteins, nucleic acids and lipids across cells. They are important mediators of cell-to-cell communication, but their relatively small size makes EV isolation and down-stream analysis challenging. In this thesis, we ventured through some of the current challenges in the EV field touching upon purification, small RNA and protein content profiling and ultimately characterization of engineered vesicles at the molecular level.

The isolation of EVs from the complex fluid they are surrounded by, represents the first hindrance in studying these vesicles. Ideally, the purification method should preserve the integrity and natural properties of the EVs and simultaneously deplete the vesicular portion from unwanted components. In Paper I, we describe a novel liquid chromatography technique for EV purification, that combines size separation with bind elution (BE-SEC) entrapping molecules smaller than 700 kDa within the matrix core. The BE-SEC isolation method yields high particles recovery in a reproducible and time-efficient way, without neither affecting the EVs natural surface protein signature nor their physicochemical properties. By adding a prior tangential flow filtration step, the BE-SEC could be scaled-up and the EV preparation further depleted from unwanted non-vesicular proteins and RNAs.

Secondly, therapeutically engineered EVs are promising delivery vehicles and linking the administered vesicular dose to the molecular cargo concentration is of extreme relevance to achieve a desired response. Therefore, analytical methods focused on single vesicles quantification rather than 'bulk' analysis and improved bioengineered vesicles are of utmost importance for therapeutic applications. In Paper II, we extensively characterize a set of fluorescently labelled EV-associated proteins, employing several qualitative and quantitative methods. Using Fluorescence Correlation Spectroscopy, we quantify the number of fluorescent molecules per single loaded vesicle. Different loading efficiencies were observed for the tested proteins, with the tetraspanins (CD63, CD9 and CD81) showing the highest loading efficiency with an average of 40-60 fluorescent molecules per vesicle. To summarize, we provide a reference for selecting EV sorting domains that best fit the desired outcome, as well as an array of quantitative and qualitative methodologies to support EV engineering.

In Paper III, we investigate the native RNA and protein content of EVs, with a focus on small RNAs and RNA binding proteins respectively. Across different mouse and human cell-derived EVs, a deficiency of miRNA sequences and relative depletion of 'miRNA-related' proteins were observed. The majority of the RNA sequences detected in EVs was represented by rRNA-, coding- and tRNA fragments, reflecting the observations in the respective protein portion, where ribosomal and translational proteins were predominantly identified.

In conclusion, this thesis explores and advances some of the challenges encountered in the EV field by ameliorating the EV isolation workflow in terms of time and scalability, linking the vesicular transcriptome and proteome of EVs derived from various cell lines, and systematically comparing and quantifying the sorting efficiency of different proteins into EVs.

# LIST OF SCIENTIFIC PAPERS

I. **Corso G*,** Mäger I*, Lee Y, Görgens A, Bultema J, Giebel B, Wood MJA, Nordin JZ, EL Andaloussi S. **Reproducible and scalable purification of extracellular vesicles using combined bind-elute and size exclusion chromatography.** *Scientific Reports. 2017 Sep 14;7(1):11561.*

II. **Corso G***, Heusermann W*, Trojer D, Görgens A, Steib E, Voshol J, Graff A, Genoud C, Lee Y, Hean J, Nordin JZ, Wiklander OPB, EL Andaloussi S, Meisner-Kober N. **Systematic characterisation of extracellular vesicles sorting domains and quantification at the single molecule – single vesicle level by fluorescence correlation spectroscopy and single particle imaging.** *In Press.*

III. Sork H, **Corso G**, Krjutskov K, Johansson HJ, Nordin JZ, Wiklander OPB, Lee YXF, Westholm JO, Lehtiö J, Wood MJA, Mäger I, El Andaloussi S. **Heterogeneity and interplay of the extracellular vesicle small RNA transcriptome and proteome.** *Scientific Reports. 2018 Jul 17;8(1):10813.*

*These authors contributed equally

# LIST OF ADDITIONAL PUBLICATIONS

IV. Nordin JZ, Bostancioglu RB, **Corso G**, EL Andaloussi S. **Tangential Flow Filtration with or without Subsequent Bind-Elute Size Exclusion Chromatography for Purification of Extracellular Vesicles.** *Methods in Molecular Biology. 2019;1953:287-299.*

V. Wiklander OPB, Bostancioglu RB, Welsh JA, Zickler AM, Murke F, **Corso G**, Felldin U, Hagey DW, Evertsson B, Liang XM, Gustafsson MO, Mohammad DK, Wiek C, Hanenberg H, Bremer M, Gupta D, Björnstedt M, Giebel B, Nordin JZ, Jones JC, EL Andaloussi S, Görgens A. **Systematic Methodological Evaluation of a Multiplex Bead-Based Flow Cytometry Assay for Detection of Extracellular Vesicle Surface Signatures.** *Frontiers in Immunology. 2018 Jun 13;9:1326.*

VI. Sork H, Nordin JZ, Turunen JJ, Wiklander OP, Bestas B, Zaghloul EM, Margus H, Padari K, Duru AD, **Corso G**, Bost J, Vader P, Pooga M, Smith CE, Wood MJ, Schiffelers RM, Hällbrink M, EL Andaloussi S. **Lipid-based Transfection Reagents Exhibit Cryo-induced Increase in Transfection Efficiency.** *Molecular Therapy Nucleic Acids. 2016 Mar 8;5:e290.*

VII. Wiklander OP, Nordin JZ, O'Loughlin A, Gustafsson Y, **Corso G**, Mäger I, Vader P, Lee Y, Sork H, Seow Y, Heldring N, Alvarez-Erviti L, Smith CI, Le Blanc K, Macchiarini P, Jungebluth P, Wood MJ, EL Andaloussi S. **Extracellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting.** *Journal of Extracellular Vesicles. 2015 Apr 20;4:26316.*

# LIST OF ABBREVIATIONS

| | |
|---|---|
| A4F | Asymmetrical-Flow Field-Flow Fractionation |
| APC | Antigen Presenting Cell |
| ARF6 | ADP-ribosylation Factor 6 |
| ARMMs | ARRDC1-mediated Microvesicles |
| ARRDC1 | Arrestin-Domain-Containing Protein 1 |
| BACE1 | Beta-site Amyloid Precursor Protein Cleaving Enzyme 1 |
| BE-SEC | Bind Elute-Size Exclusion Chromatography |
| CD | Cluster of Differentiation |
| CHMP4C | Charged Multivesicular Body Protein 4C |
| CM | Conditioned Medium |
| DAPI | 4',6-diamidino-2-phenylindole |
| DC | Dendritic Cell |
| DEX | Dendritic Cell-derived Exosomes |
| DLS | Dynamic Light Scattering |
| DMD | Duchenne Muscular Dystrophy |
| EGFR | Epidermal Growth Factor Receptor |
| ER | Endoplasmic Reticulum |
| ESCRT | Endosomal Sorting Complex Required for Transport |
| EV | Extracellular Vesicle |
| FBS | Fetal Bovine Serum |
| FCS | Fluorescence Correlation Spectroscopy |
| FDR | False Discovery Rate |
| GFP | Green Fluorescent Protein |
| GM-CSF | Granulocyte Macrophage Colony-Stimulating Factor |
| GvHD | Graft-versus-Host Disease |
| HDL | High-Density Lipoprotein |
| HEK293 | Human Embryonic Kidney 293 |
| HER2 | Human Epidermal Growth Factor Receptor 2 |
| HSPG | Heparan Sulfate Proteoglycans |
| ICAM | Intercellular Adhesion Molecule |

| | |
|---|---|
| ILV | Intraluminal Vesicle |
| LAMP2b | Lysosome-associated Membrane Protein 2b |
| LMP-1 | Latent Membrane Protein 1 |
| MFI | Mean Fluorescence Intensity |
| MHC | Major Histocompatibility Complex |
| miRNA | MicroRNA |
| mRNA | Messenger RNA |
| MSC | Mesenchymal Stromal Cells |
| MV | Microvesicle |
| MVB | Multivesicular Body |
| MWCO | Molecular Weight Cut-Off |
| NGS | Next Generation Sequencing |
| NK | Natural Killer |
| NTA | Nanoparticle Tracking Analysis |
| PBS | Phosphate Buffered Saline |
| PE | Phosphatidyl-ethanolamine |
| PEG | Polyethylene Glycol |
| piRNA | Piwi-like RNA |
| PS | Phosphatidylserine |
| PTM | Post-Translational Modifications |
| RBP | RNA Binding Protein |
| RhoA | Ras Homolog Gene Family, Member A |
| RISC | RNA-induced Silencing Complex |
| RNAi | RNA Interference |
| RNP | Ribonucleoprotein |
| rRNA | Ribosomal RNA |
| RT | Room Temperature |
| RVG | Rabies Viral Glycoprotein |
| scFV | Single Chain Variable Fragment |
| SEC | Size Exclusion Chromatography |

| | |
|---|---|
| SIMPLE | Small Integral Membrane Protein of the Lysosome/Late Endosome |
| siRNA | Small Interfering RNA |
| SNAP-23 | Synaptosomal-Associated Protein 23 |
| SNARE | Soluble NSF Attachment Protein Receptor |
| SYNCRIP | Synaptotagmin-binding Cytoplasmic RNA-Interacting Protein |
| TEM | Transmission Electron Microscopy |
| tEVs | Tumor-derived Extracellular Vesicles |
| TFF | Tangential Flow Filtration |
| TRAIL | TNF-related Apoptosis-inducing Ligand |
| Tsg101 | Tumour Susceptibility Gene 101 |
| UC | Ultracentrifugation |
| UF | Ultrafiltration |
| VAMP7 | Vesicle-Associated Membrane Protein 7 |
| VLPs | Virus-Like Particles |
| VPS4 | Vacuolar Protein Sorting-Associated Protein 4 |
| VTA1 | Vacuolar Protein Sorting-Associated Protein VTA1 |
| WB | Western Blot |
| YBX1 | Y-Box Protein 1 |
| YKT6 | Synaptobrevin Homolog |

# CONTENTS

# INTRODUCTION

## 1 Synthetic and Natural Nanoparticles as Drug Delivery Vehicles

Over the past few years, nanotechnology has insinuated into our daily lives in many different forms, from electronics to materials, from industrial to medical processes. The application of such technology for medical diagnostics and disease treatment purposes, among others, is termed nanomedicine[1]. One of the main objectives in nanomedicine, is the efficient delivery of drugs to specific diseased tissues with limited or no side effects for the healthy tissues. To achieve such effects, poorly water-soluble drugs and other therapeutical molecules are encapsulated into synthetic nanoparticles such as liposomes[2], polymer nanoparticles[3], micelles[4] and dendrimers[5] or natural carriers like viruses (virus-like particles, VLPs)[6] and extracellular vesicles (EVs)[7,8]. Synthetic nanosystems have distinct properties in terms of toxicity, biocompatibility and pharmacokinetics, which seem to be determined by the nanoparticles' formulation and physiochemical properties[1]. For therapeutic applications, the nano-delivery systems should ideally have beneficial effects over the free drug such as site-specific delivery and controlled released of the compounds, avoid recognition by and escape the body's immune system. In the last decades, the features of the nanosystems have been tuned and optimized to extend blood circulation and targeted-delivery and many nanomedicines are undergoing pre- and clinical trials and some have been clinically approved[9]. Nevertheless, the translation of such therapeutics from bench-to-bed side has been modest due to ineffective delivery to certain tissues, rapid clearance, poor understanding of the drug interaction and fate in *in vivo* settings[9]. To overcome these hurdles, naturally occurring nanosystems have been exploited and studied: viruses and EVs.

Viruses have evolved over the years to evade the immune system and transfer their genetic information into cells they infect, making their modified variants suitable for therapeutic gene delivery[10]. For safety concerns, virus-like particles (VLPs) have recently been developed. These carriers essentially constitute of virus structural proteins, but are not infectious since they lack any viral genetic material[11]. VLPs present several advantages over other nanoparticles: they are biocompatible[12], they can intrinsically encapsulate therapeutic material[13,14] and their surface can be easily functionalized with various biomolecules to provide different features[15–17]. Although many studies show the successful application of VLPs as vaccines and drug delivery vehicles, challenges in the production line, their immunogenicity and non-suitability for repetitive administrations, hinder the advancement of the VLP field towards clinical applications[18,19].

EVs are cell-derived biological carriers transporting active biomolecules between cells and tissues. They have been shown to play a fundamental role in cell-to-cell communication both in physiological and pathological conditions. Thanks to their small size and intrinsic features, EVs represent ideal candidates for drug delivery[20]. Nevertheless, they are not devoid of limiting factors that hamper their clinical translation. The characteristics, biological and therapeutical roles of the EVs will be extensively discussed in the following sections.

# 2 Extracellular Vesicles

## 2.1 History and Terminology

The discovery of cell-derived vesicles leads back to the late 1960s when researchers imaged vesicle-like material and referred to it as "platelet dust"[21]. A decade later, active shedding of vesicles was observed in lymphoma cells and it was proposed as a mechanism used by tumour cells to evade the host immune system[22]. Meanwhile, two independent groups studying the transferrin receptor, demonstrated that this receptor was secreted from reticulocytes in vesicles[23,24] and subsequently discovered that these vesicles originated from multivesicular bodies (MVBs)[25,26]. These preliminary reports helped to develop the field as we know it now and to unravel some aspects of the EV biology and their potential as therapeutics.

Since the early stages of the EV field up until now, researchers have tried to reach a consensus regarding the EV nomenclature. EVs can be catalogued based on their function[27] or specific cellular origin[28,29], but are commonly classified based on their biogenesis and biophysical properties. Exosomes are 40-120 nm in size, originate from the inward budding of the MVBs and are secreted in the extracellular environment upon fusion of the MVB with the cell membrane. Microvesicles (MVs, 50-1000 nm) and apoptotic bodies (500-2000nm) buds directly from the plasma membrane of healthy and apoptotic cells, respectively[30]. Despite this classification, there is still a grey zone where the different classes of EVs overlap in terms of size, molecular signature and density. To complicate the scenario even further, within the same class, different vesicular subpopulation with specific composition and biophysical properties have been described[31] (**Figure 1**).

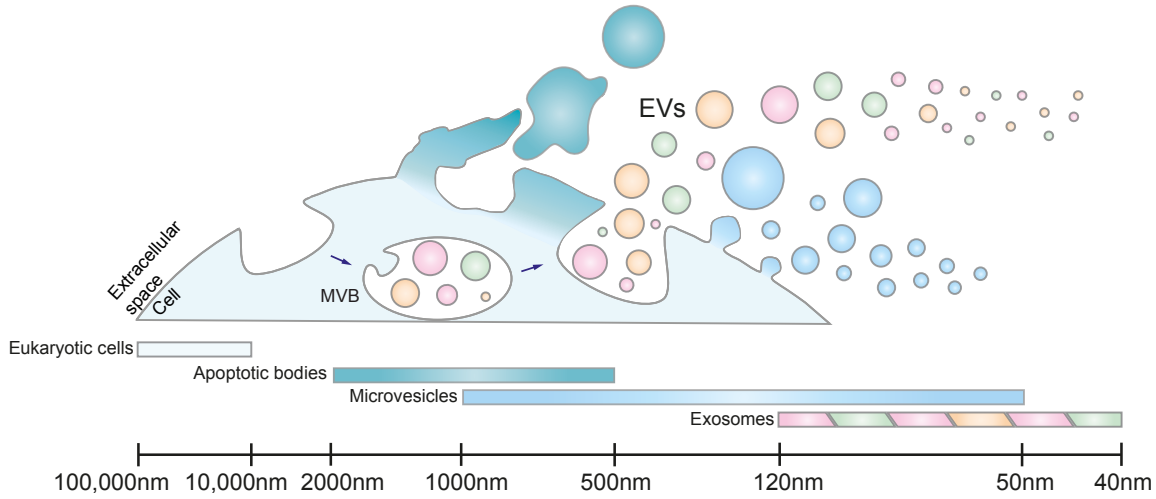


**Figure 1. Heterogeneity of the EV population.** Heterogeneous populations of EVs are release by cells through different mechanisms. Separating the different EV populations is challenging due to their overlapping sizes and characteristics. Exosomes and their subpopulations (indicated as green, pink and orange vesicles) originate from the endosomal pathway and are secreted upon fusion of MVBs with the cell plasma membrane. Apoptotic bodies (water blue) and microvesicles (light blue) buds directly from the plasma membrane. Figure inspired by[32].

For clarity, in this thesis the term EV will be referring to exosomes and microvesicles, unless otherwise stated.

## 2.2 EV Isolation

EVs are generally purified from conditioned cell culture media and various body fluids such as blood plasma[33], urine[34], saliva[35], breast milk[36], semen[37], amniotic fluid[38] and many more. These fluids are complex and contain, apart from EVs, cell debris, proteins, lipoproteins and nucleic acids among others. Therefore, the isolation of EVs is challenging, not only due to their small size, but due to the complexity of the material they are surrounded by.

### 2.2.1 Non-technical Related Considerations

Mammalian cells are commonly cultured in medium supplemented by fetal bovine serum (FBS), but the presence of serum-derived vesicles poses a risk of contamination that might influence the experimental outcomes. Therefore, to minimise the influence of FBS-derived EVs, it is recommended to culture cells in serum-free conditions or using EV-depleted FBS[39,40]. In addition, FBS contains diverse RNA species, either vesicle- or protein-associated, that are only partially removed by the common EV-depletion methods and could contaminate and interfere with downstream analysis and applications[41]. Cell culturing conditions such as hypoxia[42], serum starvation[43], three-dimensional culturing[44] were also shown to impact the secretion, composition and function of the EVs.

Biological fluids are even more complex than cell culture media due to the presence of free components such as proteins, carbohydrates, lipids and nucleic acids that make them relatively viscous. For this reason, it is advisable to dilute them in equal volumes of phosphate buffered saline (PBS) prior to any purification procedure[39] and consider important factors during sample collection, handling and EV isolation[45,46]. These considerations are fundamental to avoid co-isolation of contaminants that might impact on data interpretation. For instance, high-density lipoproteins (HDL) contained in blood plasma, have been shown not only to co-purify with EVs owing to density similarity, but to carry a distinct miRNA signature and mediate biological effects in recipient cells[47,48]. Henceforth, in order to correctly interpret EV-mediated effects, it is fundamental to acknowledge the existence of such hurdles and tailor the isolation method to reduce the contamination to a minimum.

The loss of EVs due to binding onto the storage vials, vesicular disruption due to repetitive freeze-thaw cycles[49], or lysis due to the storage buffer of choice[50] are all aspects that have an impact on the experimental outcomes and should be considered when storing isolated EVs. The lack of knowledge on EV preservation prior and after isolation are stalling the progression of EVs as therapeutics. Therefore, decrypting the optimal EV storage conditions to preserve the EV characteristics is vital for clinical applications.

In conclusion, all these potential issues during culturing, isolation and storage have to be taken into consideration while outlining, performing and interpreting experiments.

### 2.2.2 EV Isolation Methods

There are different aspects that should be taken into consideration while isolating EVs: ideally the procedure should result in high particle yields and depletion of any extra-vesicular

contaminants while maintaining the integrity and biophysical properties of the vesicles. A perfect isolation method does not exist, but progress has been made to develop improved isolation techniques.

The isolation of cell cultured derived-EVs can be achieved via different methodologies, but all of them share the same pre-clearance steps. Floating cells and cell debris are depleted at low-speed centrifugations, 300 x *g* and 2,000 x *g* respectively[39]. The isolation of EVs from biological fluids is similar, but the speed and length of the centrifugation steps are usually increased due to the viscosity of the sample[39]. Additional details of the most commonly used EV isolation methods are provided below.

#### *2.2.2.1 Ultracentrifugation*

Ultracentrifugation (UC) is the most utilized EV isolation technique in the field[39,51]. The low-speed centrifugation steps described above are most often followed by a 0.22 μm filtration or by an optional 10,000-20,000 x *g* spin to either filter out or pellet bigger vesicles i.e. microvesicles (MVs). The supernatant is then spun at 100,000-120,000 x *g* to enrich for small vesicles termed exosomes. The purity of the preparation can be further enhanced by introducing a PBS wash[39] or by loading the pelleted vesicles on a density gradient to separate the different components of the secretome[39,52] and EV populations[31] based on their buoyant density.

Despite being extensively used, UC has limitations such as low particle yields, particle disruption and aggregation, co-pelleting of non-vesicular biomolecules, operator-dependence, long isolation time and poor scalability[53,54]. Even the additional density gradient step leads to particle disruption and loss of EV functionality[55], likewise the spinning time and the rotor type affects the purity and yield of the final sample[56]. Therefore, to overcome these hurdles, several alternative isolation methods have been developed.

#### *2.2.2.2 Size exclusion chromatography*

Separating EVs based on their size is becoming a popular alternative to UC, since it does not seem to affect the integrity of the EVs[53,57] and results in higher yields and purer particles[52,53]. Size exclusion chromatography (SEC) is performed using columns filled with porous polymer beads of different sizes. As the solution travels across the column, big molecules pass through the pores faster and elute earlier than smaller molecules, which can enter the porous beads and thus have a longer retention time. Studies adopting this methodological principal and the proper column resin, have described the feasibility of SEC in fine fractionating the secretome[53,58] and separating different EV subpopulations[31]. However, the technique in itself is not scalable, a fundamental aspect for clinical applications, and is therefore commonly combined with prior Ultrafiltration (UF)[53,58] or Tangential Flow Filtration (TFF)[31,59,60] steps to concentrate large media volumes. Both methods use semipermeable membrane with defined molecular weight cut-offs (MWCO, typically 100 or 300 kDa), UF being based on dead-end and TFF on cross-flow filtration. In essence, molecules bigger than the MWCO are retained either in the filter or hollow fibres and smaller molecules elute in the permeate.

Commercially available columns based on size exclusion have also been adopted to reduce the isolation time[59] and to allow the purification of EVs from small volumes of biofluids[61,62].

#### *2.2.2.3 Alternative Isolation Methods*

Based on the diverse physiochemical and molecular EV characteristics, several alternative isolation procedures have also been developed.

The heterogeneous physical characteristics of EVs such as hydrodynamic diameter and molecular weight drive their separation in asymmetrical-flow field-flow fractionation (A4F)[63,64]. Even though this technique requires substantial optimization, it preserves the native EV characteristics and performs a gentle separation of EVs into subpopulations, enabling the discovery of a novel small non-membranous particle population termed exomers (~35 nm)[65]. Some other methodologies exploit the expression of certain markers on the EV surface to perform immunoaffinity capture[66,67]; even though multiplexed, these techniques tend to preferentially isolate certain EV subtypes based on their surface protein profile or isolate unwanted membranous material that expresses similar antigens.

Commercially available and easy-to-use products have also been developed, such as ExoQuick™ (System Bioscience) and Total Exosomes Isolation™ (ThermoFisher Scientific). Both are polymer-based precipitation techniques and although being facile to use, the low purity and the residual polymers represent an issue for downstream applications[68,69]. Despite these disadvantages, polyethylene glycol (PEG)-precipitation has been employed to isolate EVs further administered to a patient and seemed to be well tolerated[70].

To conclude, the lack of consensus on the most ideal isolation routine, has an impact on the reproducibility and reliability of the findings, making the cross-study comparison more challenging. However, efforts towards more standardized experimental reporting to facilitate the interpretation and reproducibility of the studies have been made[71].

## 2.3 EV Characterization

The vesicular characterization following isolation is essential, not only to determine and confirm the presence of EVs in the preparation[72], but also to acquire information of their cargo and its potential implication in biological processes. Therefore, the development of novel characterization methods to study the physiochemical and molecular properties of the EVs is as important as the advancement of the purification protocols. The technical challenges associated with the isolation protocols, are also reflected in the characterization procedures. Therefore, features such as size, morphology, density, concentration and molecular content are commonly and jointly utilised for EV characterization and will be shortly described below.

### 2.3.1 Physical Characterization

Transmission electron microscopy (TEM) was firstly used to image what we nowadays refer to as EVs[26] and it is still widely utilized to visualize and characterize EVs with high resolution. However, the specimen preparation and acquisition may damage the sample and introduce artefacts that hamper proper characterization. Hence, the visualization of EVs in their native state, preventing morphological and ultrastructural changes, has been achieved by embedding the sample in vitreous ice and keeping it at low temperatures with cryo-EM[73]. Additionally, both methodologies can be applied to collect molecular information by staining the specimens with immunogold conjugated antibodies directed towards specific EV antigens[74,75]. Recently developed microscopy-based techniques such as atomic force microscopy (AFM), have been seldomly applied to physically characterize EVs, but shown to be promising[76]. The AFM outputs a 3D-topography of the EVs, immobilized on a flat surface of mica that can be further functionalized with monoclonal antibodies to obtain information on the EV surface proteome[77].

Beside the microscopy-based techniques, other methodologies are widely adopted in the EV field. For instance, the size and distribution of particles in solution can be determined by the particles' Brownian motions. Dynamic light scattering (DLS) employs this physical phenomenon to measure particles ranging from 1 to 6000 nm, however in the presence of an heterogenous suspension, the particle distribution tend to be skewed toward the larger particles, influencing the results[78]. The heterogeneity of the population can be more accurately measured with nanoparticle tracking analysis (NTA) allowing for the detection of particles as small as 30 nm[51,79]. NTA offers quick and easy measurements, but the particle concentration has to be within a certain range to achieve accurate results. Fluorescently labelled vesicles can be detected either by NTA, even though accuracy can only be achieved with very bright vesicles[80], or by fluorescence correlation spectroscopy (FCS), a sensitive method able to quantify the number of fluorescent molecules per vesicle[81] (Paper II).

EVs can also be characterized and classified based on their density in sucrose or iodixanol (OptiPrep™) gradients[39]. Exosomes have been reported to have a buoyant density ranging from 1.13 to 1.19 g/ml[82], whereas MVs around 1.03-1.08 g/ml[83]. This procedure has also allowed the detection and separation of EV subpopulations characterized by different flotation densities[31].

### 2.3.2 Molecular Characterization

Physical vesicular features are normally coupled to the characterization of the EV molecular content, in particular the EV protein composition. Total protein assays are most frequently used to define the purity of the isolated vesicles[52] and determine the doses for *in vitro* and *in vivo* studies. However, they are limited to the purity of the sample as protein contaminants compromise the accuracy of the measurements. On the other hand, the detection of specific proteins by immunoblotting is widely used to validate the expression of EV-associated proteins (e.g. cluster of differentiation (CD)9, CD81, CD63, Alix, Tsg101) and absence of contaminating proteins (e.g. GM130, Calnexin, Albumin, Fibronectin)[72,84] in the isolated samples. Other than being used as a quality control tool, immunoblotting can be applied to

detect exogenous or disease-specific proteins. As a complement or substitute for WB, EV surface markers detection by flow cytometry and relative protein expression determination, can be achieved employing multiplexed beads coated with antibodies directed toward 39 different antigens[66,67]. Nowadays, to overcome some of the flow cytometry limitations[85], more sensitive flow cytometers have been developed to quantify and characterize EVs as small as 100 nm, upon staining with immunofluorescence antibodies[86] or coupling with imaging-quantification features[87–89]. High-throughput proteomic studies have also been employed to discover novel EV markers[90], compare the EV proteome upon different isolation methods[91] or discriminate between EV subpopulations[31].

Since the discovery of EV-mediated RNA transfer[92], the characterization of nucleic acids in EVs has expanded. The RNA content is generally investigated by next generation sequencing (NGS) or microarrays, with further validation by qPCR or northern blotting. The presence of contaminants such as lipoproteins or ribonucleoproteins (RNPs) originating from the cell culture media[40,93,94] or DNA carry-over from RNA extraction[95] might negatively affect the downstream analysis. Hence, to reduce the contaminations it is recommended to treat the EV preparation with proteinase, RNase or DNase to eliminate any extravesicular component[95]. However, the impact of these treatments on the nucleic acids associated with the EV surface is still unknown. An additional challenge is the assessment of the quantity and quality of the RNA required prior to the NGS analysis, due to the low RNA content per EV and low sensitivity of the currently available assays[95].

Even though neglected for a long time, the lipid content of the EVs is gaining attention. The employment of gas liquid chromatography[96,97] or mass spectrometry[98], has shown the enrichment of cholesterol, glycolipids, sphingomyelin and phosphatidylserine in EVs as compared to the cells of origin. More recently, the lipid component of the EVs has been adopted to develop a lipid quantification assay devoid of the limitations owed to the protein aggregates[99].

The small size, the heterogeneity, the presence of contaminants and the poor sensitivity of the current technologies, make the EV characterization still relatively challenging.

## 2.4 EV Composition

EVs are vesicles composed by a lipid bilayer that encloses proteins and nucleic acids, protecting them from degradation. Numerous high-throughput studies on the lipidome, proteome and transcriptome of these vesicles have been performed to date and compiled on web-based catalogues such as ExoCarta and Vesiclepedia. These studies have revealed that the nature of the EV content is highly dependent on the source cell and its physiological or pathological state. Nevertheless, common features are shared between the different vesicles and their subpopulations[100–102].

### 2.4.1 Lipids

The interest in studying EV lipids has been growing since 2002, when it was demonstrated for the first time that lipids of tumour-derived EVs, particularly sphingomyelin, played a key role in angiogenesis[103]. Albeit the differences related to the cell type of origin, EVs are mainly composed of phosphatidylserine (PS), phosphatidyl-ethanolamine (PE), sphingomyelin, phosphatidylinositol, phosphatidylcholine, cholesterol and ceramides (GM3)[96,98,104–106]. It is well established that the inner and outer leaflets of the EV membrane have an asymmetric distribution of lipids that regulate the curvature of the membrane. In this regard, the most notable difference as compared to the cell plasma membrane is the presence of PS and PE on the outer leaflet of the exosomal membrane that seem to have a role in exosomes biogenesis[107]. The enriched lipids, especially sphingomyelin and GM3, has been shown not only to convey stability and structural rigidity to the vesicular membrane[108], but also to take part in cellular signalling pathways[109].

### 2.4.2 Protein Composition and Sorting Mechanisms

Proteins are a major component of the EV cargo and their expression is commonly used for characterization purposes. A compilation of 16 proteomic data sets has identified a collection of recurring proteins and categorized them as common vesicular markers, accepted across the EV community[72,84]. Many of these EV-associated proteins are regulators of the EV biogenesis. Proteins of the tetraspanin family (CD9, CD81 and CD63) are highly enriched on exosomes[110–112], but not exclusive to exosomes as they are also detected on bigger vesicles[90]. The abundance of the tetraspanins on EVs is the result of the formation of clustered microdomains together with other partners such as integrins[113,114] and syntenin[115], that promote the budding of the membrane either towards the extracellular environment or toward the lumen of the MVBs[112,116]. Similarly, proteins of the Endosomal Sorting Complex Required for Transport (ESCRT) involved in MVB biogenesis, such as Tsg101[15] and Alix are frequently found in exosomes. The latter was shown to interact with syndecan through syntenin, supporting exosome biogenesis and ensuring cargo loading into the vesicles[118]. Various post-translational modifications (PTM) are also known to control protein localization, stability, activation state and sorting into EVs[119,120]. For instance, the recognition of the ubiquitinated epidermal growth factor receptor (EGFR) by the ESCRT complex, promotes the invagination of the endosomal membrane and thus the recruitment of EGFR into EVs[97]. On the contrary, the ubiquitination of small integral membrane protein of the lysosomes/late endosome (SIMPLE)[121] or major histocompatibility complex (MHC) class II[122] has a negative impact on the secretion of these proteins into EVs. The phosphorylation state of certain proteins has been shown to influence their sorting in EVs, by protecting them from endosomal degradation, as in the case of Annexin A2[123].

Despite the predominance of common proteins, EVs also contain cell-type specific proteins like MHC class II, very abundant on antigen presenting cells (APCs)-derived vesicles[124–126] and EGFRvIII only identified in EVs derived from glioma cells[127]. Additionally, the most abundant cellular proteins (e.g. actin, tubulin etc.) have also been detected in EVs and this could

be explained by bulk inclusion of cytoplasmic material into EVs or more likely due to contaminants and fragments of dying cells carried over during isolation.

### 2.4.3 Nucleic Acid Composition and Sorting Mechanisms

Roughly ten years ago, a series of publications described the EV-mediated functional transfer of mRNAs and miRNAs between cells[92,128–131]. Since then, numerous studies based on microarray and next generation sequencing have profiled the RNA content of EVs derived from various cells[132–136] and biological fluids[137,138]. Double stranded DNA[139,140], mitochondrial DNA[141] and dsDNA-binding histone proteins[142] have been detected in EVs, however their association with EVs is disputable as DNA is still considered a contaminant from improper isolation[95,143]. Recently, the secretion of DNA and histones was shown to be an autophagy/amphisome-dependent mechanism and no association with exosomes was observed[143]. However, more studies are needed to elucidate the genuine role of DNA in EVs.

EVs are mainly enriched in transfer RNAs (tRNAs), miRNAs, small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), vault RNAs (VT-RNAs), Y RNAs and fragmented RNAs[144–146]. Overall, the EV RNA content reflects that of the parental cells but certain RNA species are more overrepresented than others, indicating a certain degree of sorting specificity. *In silico* studies have identified specific 'zipcodes' sequences[147,148] and 3'UTR regions[144] in the mRNA fragments loaded into EVs, supporting an active RNA sorting mechanism. RNA binding proteins (RBPs) such as hnRNPA2B1[149] and SYNCRIP[150] have also been identified as RNA sorting machineries leading to the enrichment of miRNAs in EVs, through the recognition of specific motifs. Similarly, using a cell-free assay, Shurtleff and colleagues demonstrated the role of the protein YBX1 in binding and sorting miRNAs into exosomes[151]. The interaction between Exportin-5, a protein involved in exporting pri-miRNAs from the nucleus to the cytoplasm, and ADP-ribosylation factor 6 (ARF6) were reported to have a direct role in trafficking pre-miRNAs into tumour-derived MVs[152].

Due to the observed association of miRNAs and RNA-induced silencing complex (RISC) proteins with MVBs[153], it seems reasonable to foresee an involvement of these proteins in miRNA sorting. Some reports, in fact, described the presence of Ago2 and other RISC proteins in breast[154]- and colon[155] cancer-derived EVs, further revealing a sorting dependency related to Ago2 phosphorylation state[155,156]. In relation to this, Ago2 knockout was shown to decrease the sorting of certain miRNAs into HEK293T-exosomes[157]. We observed that, despite overexpression, the native isoform of Ago2 was not sorted into HEK293T-EVs, unless fused to a membranous tag which further resulted in an enhanced vesicular loading of certain miRNAs (unpublished data). Despite these observations, the presence of Ago2 in EVs is still controversial as some studies have reported little or no Ago2 detection in the vesicles[143,158,159]. Hence, further studies are required to elucidate the mechanisms behind RNA sorting in EVs and the potential involvement of RISC proteins. These observed discrepancies could be confined to the cell culture conditons[93,94] or the isolation methods[159] utilised.

## 2.5 EV Biogenesis

Microvesicles are generated from the outward budding of the plasma membrane and released in the extracellular environment[160]. Exosomes, on the other hand, originate as intraluminal vesicles (ILVs) from the inward budding of the MVBs, which then fuse with the cell membrane and secrete the enclosed vesicles into the extracellular space[161] (**Figure 2**). Despite these traditionally accepted biogenesis processes, evidence of vesicles carrying similarities with exosomes, have been described to bud from the plasma membrane of T-cells[107]. To determine whether this is a cell-type specific or a general mechanism that has not yet been explored, further investigations are required.

### 2.5.1 Biogenesis of Microvesicles

Microvesicles are directly released in the extracellular milieu by blebbing and scission of the plasma membrane[162]. Several rearrangements of the lipid and protein composition are required to perturb the rigidity and curvature of the membrane during the MV formation[160]. Aminophospholipid translocases (flippase and floppase) contribute to the translocation of phospholipids between the two membrane leaflets, essential in the first steps of the MV formation[163,164]. Upon lipid redistribution, the fission and release of the MVs from the cell are led by the cytoskeletal rearrangements of actin and myosin, regulated by a signalling cascade initiated with ARF6 and RhoA mediators[160,165]. External factors such as $Ca^{2+}$ levels[166] and hypoxia[42] have also been shown to activate and influence MV formation. Recently, other types of EVs have been described to bud directly from the plasma membrane, termed arrestin-domain-containing protein 1 (ARRDC1)-mediated MVs (ARMMs), which were shown to lack late endosomal markers and contain plasma membrane associated ARRDC1 and Tsg101, driving their direct secretion[167,168].

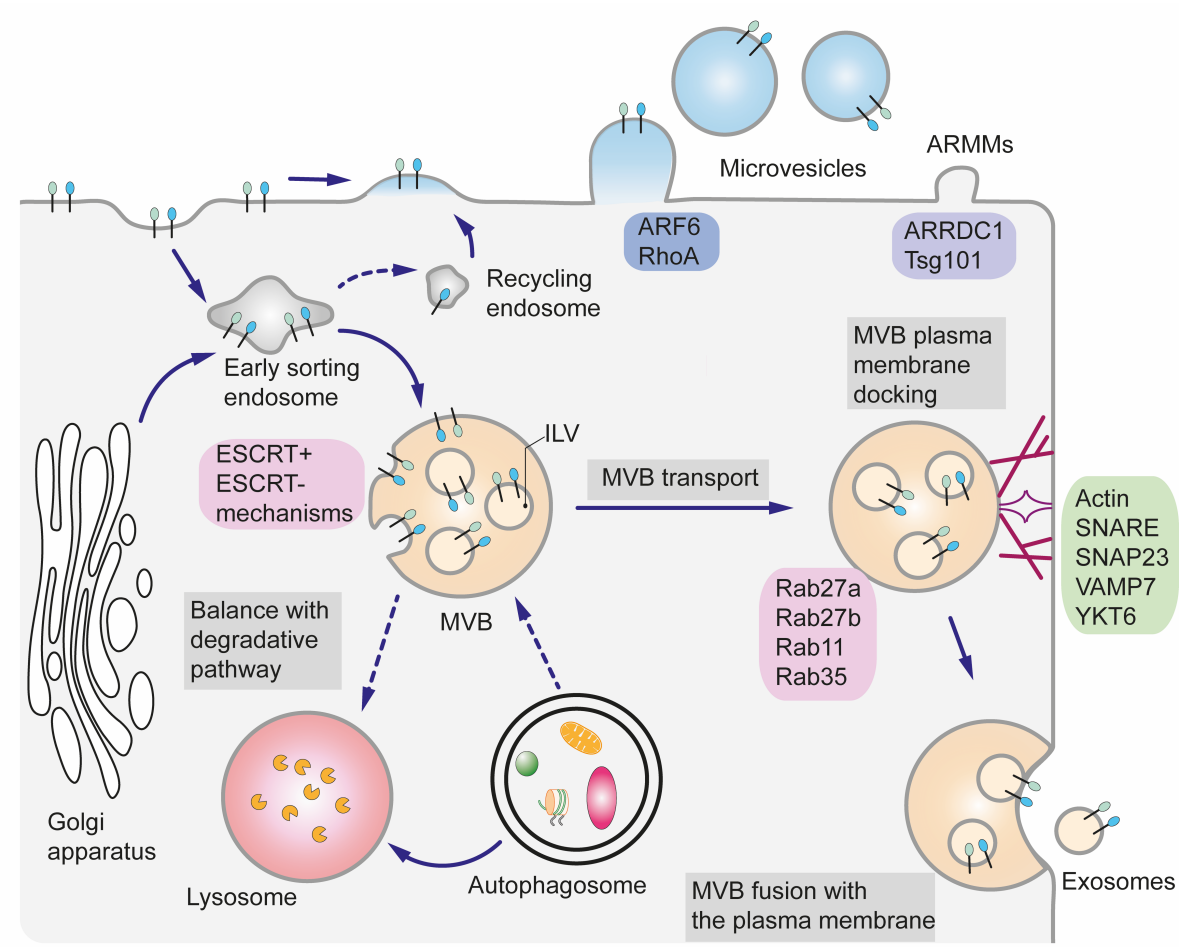


**Figure 2. EV biogenesis.** The budding of microvesicles from the plasma membrane is controlled by the proteins ARF6 and RhoA, regulators of the cytoskeletal elements. Recently, other types of MVs (ARMMs) were shown to directly originate from the plasma membrane by ARRDC1 and Tsg101 intervention. Exosome biogenesis is more complicated and involves the endosomal pathway. The cargo directed to the MVB originates from the invagination of the cell plasma membrane forming the early sorting endosome. The cargo contained in the early endosome can be recycled back to the cell plasma membrane through the recycling endosome or directed to the MVB. The MVBs are compartments filled with ILVs formed from the invagination of the MVB membrane through ESCRT-dependent and -independent mechanisms. MVBs can either degrade their content by fusing with the lysosome, fuse with the autophagosome generating a hybrid amphisome directed to the plasma membrane or be transported directly to and fuse with the plasma membrane. The docking and fusion to the plasma membrane are regulated by actin, Rab and SNARE proteins. Figure inspired by[169]. *ARMMs, arrestin-domain-containing protein 1 (ARRDC1)-mediated MVs; ESCRT, Endosomal Sorting Complex Required for Transport; ARF6, ADP-rybosilation Factor 6; RhoA, Ras Homolog Gene Family Member A*.

### 2.5.2 Biogenesis of Exosomes

The biogenesis of exosomes is closely related to the endosomal pathway, that has different trafficking checkpoints to regulate the recycling of proteins to the cell membrane, to the Golgi apparatus or to the ILVs. The first step in the exosomes biogenesis, is the formation of the early endosome from the invagination of the plasma membrane that allows the internalization of specific proteins localized on the cell surface and the entrapment of extracellular components[170]. Proteins that are destined for recycling are translocated back to the plasma

membrane and will therefore not end up in ILVs, unless their endosomal recycling is impaired as for the transferrin receptor in reticulocytes[171]. Syntenin for instance, acts as a recycling and intraluminal sorting mediator. In fact, it plays a dual role by recycling syndecan to the plasma membrane via the interaction with phosphoinositide PIP2[172] or by sorting syndecan into EVs via the ESCRT mediator Alix[118,173].

During maturation of the endosomes, the ILVs are formed through the invagination of the MVB membrane. This process is regulated by the ESCRT complex composed of four different subcomplexes (ESCRT-0, -I, -II, -III) and accessory proteins (Alix, VPS4 and VTA-1)[174,175]. Ubiquitinated proteins seem to recruit ESCRT-0 and ESCRT-I, initiating the clustering and clathrin coating of the cargo proteins, leading to invagination of the endosomal membrane. The budding and scission of the membrane are finalized by the subcomplexes ESCRT-II and -III, along with the protein VPS4[176,177]. An RNA-interference screening, targeting 23 ESCRT and ESCRT-associated proteins, identified 7 proteins that influence exosome biogenesis[178]. Depletion of proteins of ESCRT-0 or -I subcomplexes such as HRS and Tsg101 reduced exosome release, whereas knockdown of CHMP4C, VPS4, VTA-1 and Alix resulted in an increased exosome secretion. Many other reports have adopted the RNAi strategy to study the biogenesis of exosomes, revealing alternative ESCRT-independent biogenesis pathways that may act in synergy with the ESCRT complex and are not mutually exclusive. Alternative pathways for ILVs formation were shown to require the sphingolipid ceramide[97] or the protein CD63[179].

#### *2.5.2.1 Exosome Secretion*

MVBs are primarily destined to fuse with lysosomes in order to degrade their content. However, regulatory mechanisms that prevent the degradation and promote ILVs secretion exist. Several studies have supported the hypothesis of a balance between degradation and secretion to maintain the cellular homeostasis, but the mechanisms are still largely unexplored. For instance, the impairment of lysosomal activity via inhibition of the endosomal proton pump was shown to increase EV secretion[180,181]. Similarly, in certain diseases where the lysosomal function is compromised leading to the accumulation of proteins or lipids in the endosomal system, triggered the secretion of those accumulated components in EVs[182]. Emerging evidences seem to suggest a similar balance between exosomes secretion and autophagy, a process where proteins and damaged organelles are captured within the autophagosome and degraded by fusing with the lysosome[183]. Sorting regulators like Tsg101 seem to have a role in tuning this balance: when subjected to the PTM ISGylation, Tsg101 inhibited exosome release by promoting selective autophagy or otherwise enhanced exosome secretion when the ISGylation site was mutated[181].

The transport and fusion of the MVBs towards the plasma membrane is promoted and regulated by the association of the cytoskeleton with specific Rab GTPases and SNARE proteins[184]. Several Rab proteins were shown to be involved in the secretion of exosomes from different cell types. For instance, Rab11 is required in the exosome pathway of K562 cells[185] and Rab35 in oligodendroglial cells[186]. Upon knocking down Rab2b, Rab9a, Rab5a, Rab27a and Rab27b

in HeLa cells, Ostrowski and colleagues observed an intracellular accumulation of endosomal vesicles and an impairment of exosome secretion, yet no influence on the normal secretory pathway of soluble proteins[187]. Several other studies have confirmed the reduction of exosome release upon Rab27a silencing, which has become a common strategy to modulate exosome secretion[188,189]. The final step for exosome secretion involves the docking and fusion of the MVBs with the plasma membrane, mediated by SNARE proteins[190]. The SNARE proteins VAMP7[191], YKT6[192] and SNAP23[193] were identified as key players in the secretion of exosomes in K562, HEK293 and HeLa cells, respectively. Cytoskeletal proteins also play a role in MVB trafficking and docking. For instance, the actin regulatory protein contractin was shown to regulate actin stability and exosome secretion, mediating its effect via the interaction with Rab27a and coronin1b[194].

Most of the studies describing the involvement of protein regulators in the exosome secretion pathway, are derived from RNA interference analysis that do not take into consideration the perturbation caused on the overall cellular level.

## 2.6 EV Uptake

Upon secretion into the extracellular milieu, an interaction between the EVs and the surrounding cells has to occur, in order to promote the release of vesicular cargo and elicit a response in recipient cells. The EV uptake has been linked to a plethora of different endocytic mechanisms, including phagocytosis, macropinocytosis, clathrin-dependent endocytosis and mere fusion with the plasma membrane. The involvement of several uptake pathways is likely to reflect the heterogeneity of the EV population linked to the different molecular surface signatures and to the downstream effects prompt by the vesicles.

The first step for EV internalization is the docking to the recipient cell surface through different mediators like integrins and proteoglycans. Integrins on the surface of the EVs have been shown to interact with intercellular adhesion molecules (ICAMs) expressed on the surface of dendritic cells (DCs)[195]. The organ tropism of cancer derived EVs i*n vivo* and their ability to promote premetastatic niche formation, were described to be dependent on the integrin signature of the EVs[196]. Integrins, in particular α4 and β4 chains, complexed with Tetraspanin8 on the exosomal surface, resulted in a selective uptake by endothelial and pancreatic cells[197]. Heparan sulfate proteoglycans (HSPGs) expressed on the cell surface, are one of the most utilized route of internalization employed by nanoparticles[198]. They were also shown to promote the uptake of cancer cell-derived EVs, inhibited by the addition of heparin, an heparan sulfate mimetic competing with HSPGs[199]. The enrichment of PS on the surface of the EVs have also been shown to facilitate macrophage internalization through phagocytosis[200,201].

Upon interaction with the cell membrane, EVs are usually internalized by the energy-dependent process of endocytosis[199,202–204]. The term endocytosis includes a number of different internalization mechanisms and all of them have shown some sort of involvement in EV uptake[205]. The inhibition of dynamin2, a protein that promotes the scission of newly formed

clathrin-coated vesicles, was shown to prevent EV internalization in phagocytic cells[206]. However, several studies have demonstrated a lack of involvement of clathrin-dependent endocytosis[203,207]. Similar discrepancies were observed with the suppression of caveolin-1 that resulted in increased EV uptake in embryonic fibroblast cells[207], but had no effect in HeLa cells[203]. Analogously, studies on the macropinocytosis mediated uptake revealed positive[203,204,208] and negative[206] association with EV internalization. A recent study revealed that filopodia drive EV uptake at specific endocytic spots. Once internalized, the EVs contained in endocytic vesicles were trafficked to the ER before fusing with the lysosomes[202]. Interestingly, the ER has also been described as a site of nucleation for the RISC complex[209], indicating a potential pathway responsible of the regulatory effects observed in recipient cells, upon EV-mediated delivery of miRNAs and siRNAs. Independently of the mechanisms of entry, EVs access the endosomal system via early endosomes which are destined to degradation in the lysosomes. This pathway would hinder the delivery of the EV cargo, but due to the numerous EV mediated functional effects observed in cells, mechanisms that regulate the EV endosomal escape are likely to exist. A possible way of avoiding the endosomal system and deliver the cargo directly into the cytosol, is via the direct fusion of the EV membrane with the cell. However, very little evidences have supported the EV uptake by fusion, a phenomenon only observed and described in cancer cells under acidic conditions[210].

### 2.6.1 Tracking EV Uptake *in vitro* and *in vivo*

The visualization of EVs *in vitro* and *in vivo* typically requires the use of fluorescent lipophilic dyes that stain the EV membrane. Dyes like PKH-67[86,189,211], PKH-26[212,213], DiO[214], DiD[215], DiR[8,216] and CFSE[217,218] are widely used to label EVs post-isolation. However, they do not exclusively stain EVs, but all membranes and lipid-rich particles present in the sample[219,220]. Due to their lipophilic nature, these dyes can also be transferred from the EVs to the cell membrane, leading to a misinterpretation of the biodistribution and localization of the vesicles[221,222]. Moreover, in the presence of salt-containing buffers, these lipophilic stains can form micelles that are difficult to discriminate from the 'real' vesicles and provide additional artefacts[219]. Efforts have been made to overcome these issues by fusing EV associated proteins with reporter tags within the cells[88,202,221,223–225]. Nonetheless, both strategies require modifications of the natural EVs and whether these changes affect the EV biodistribution or uptake, remain to be elucidated.

## 2.7 Biological and Pathological Roles of EVs

Despite the vast literature covering the roles of EVs in pathological conditions, there are some evidences suggesting the involvement of EVs in the maintenance of normal physiology. Studies on the immune system have shown that EVs derived from several immune cells, contain molecules typical of the immune system such as MHC-II molecules[226], interleukin 15 receptor α-chain[227], CD86 and ICAM-1[228]. All these molecules have an impact on different immunological functions including induction of antigen-specific T cells response[228–230],

promotion of natural killer (NK) cell proliferation[227] and DCs maturation[231]. EVs also play a role in neuronal communication[232] and promote axonal regeneration *in vitro* and *in vivo* upon sciatic nerve injury[233]. An implication in stem cell plasticity has also been confirmed, indicating that stem cell EVs have a role in regenerating injured tissues[234,235]. Despite these studies, the physiological roles of EVs remain elusive owing to the challenges in studying endogenous vesicles *in vivo*. However, efforts towards developing *in vivo* models to unravel EV physiology have been recently made[236].

More clarity has been reached in the context of disease pathogenesis. The implications of EVs in tumor biology have been extensively investigated, suggesting a role in tumor progression by promoting angiogenesis[237], pro-metastatic processes[189] and facilitating immune escape[238]. The expression of the apoptotic molecules Fas Ligand[239,240] and TNF-related apoptosis-inducing ligand (TRAIL)[241] on tumor-derived EVs (tEVs), was shown to promote tumor progression by inducing T-cell apoptosis[238]. Epstein-Barr Virus associated tumours release EVs expressing the latent membrane protein 1 (LMP-1) allowing the tumor to escape the immune system[242]. Additionally, tumor cells release EVs to prime specific tissues to establish a metastatic niche. For instance, pancreatic ductal adenocarcinoma cell-derived EVs were reported to initiate a premetastatic niche in the liver[243], whereas EVs derived from highly metastatic melanoma cells were able to recruit bone marrow derived cells to the pre-metastatic site[189]. Beyond tumors, EVs are found to be involved in the spread of neurodegenerative diseases. In Alzheimer's disease, EVs carrying β-amyloid, the toxic protein responsible for the formation of amyloid plaques, promoted its deposition in several areas of the brain[244,245]. Similarly, EV-associated α-synuclein, probably enables the progression of Parkinson's disease[246]. EVs were also found to carry host-encoded prion proteins (abnormally folded proteins) that due to their accumulation in neuronal cells cause fatal neurodegenerative disorders[247,248].

Furthermore, biofluid-derived EVs have also been exploited for non-invasive diagnostic and prognostic purposes. A number of studies on tumour-derived EVs, have revealed an altered RNA signature[130,249–254] and protein composition[130,196,255] of the vesicles, proposing an implication of these molecules in different stages of cancer progression. Therefore, the proteins and nucleic acids associated with the EVs, known to reflect the pathophysiological state of the source cell, could be employed as potential diagnostic biomarkers for the detection of primary tumours, metastasis and cancer progression in response to therapies[130,137,253,256,257]. Additionally, in order to support the advancement of EVs as liquid biomarkers towards the clinic, a number of microfluidic devices have been developed to efficiently immune-capture EVs expressing certain disease-related proteins and provide an easy-to-use detection tool[258–262].

All these observations on the involvement of EVs in physio-pathological processes, suggest that EVs could be harnessed as therapeutic carriers for tissue regeneration, immunomodulatory therapies, tumour vaccination and exogenous molecules, and as therapeutic targets to hinder EV-mediated pathogenesis.

## 2.8 Therapeutic Potential of EVs

EVs have the inherent ability of transporting different macromolecules over long distances and deliver those messages into cells triggering a response[92,263], suggesting a potential application as therapeutic agents. The EV-mediated effects can be determined by different factors like cell source of origin, surface molecules and potential EV manipulations that enhance intrinsic features of EVs. All these aspects will be described hereafter.

### 2.8.1 Innate Therapeutic Potential of EVs

Mesenchymal stromal cells (MSCs) isolated from bone marrow and adipose tissue have been thoroughly studied due to their immense therapeutic potential[264–266]. MSCs have been employed in tissue regeneration to restore damaged tissues and organs[267] or to treat immunological diseases due to their immunomodulatory properties[268–272]. Despite reporting beneficial effects, MSCs sparsely engraft *in vivo*. Hence, it was hypothesised that factors secreted by MSCs could be responsible for the observed effects. This hypothesis was strengthened when the first studies, employing the MSC secretome, demonstrated beneficial effects[273–275]. The regenerative and immunomodulatory activity of the secretome was attributed to the EVs rather than the extravesicular fraction[274], opening up a whole new world of potential EV therapeutic applications. From that moment onwards, a myriad of studies have pre-clinically demonstrated the potential of MSC-EVs in treating various pathological disorders like acute kidney failure[276,277], myocardial infarction[273,274,278], liver injury[279,280] and perinatal asphyxia[281]. MSC-EVs were also clinically administered to a steroid-refractory graft-versus-host disease (GvHD) patient, resulting in an improvement of the clinical GvHD symptoms up to 4 months after treatment[282]. Despite the beneficial effects observed upon MSC-EVs administration, recent studies have shown a therapeutic immunosuppression in GvHD mouse models, mediated by *in vivo* apoptosis of injected MSCs triggered by cytotoxic T cells[283].

Other MSC sources have also been under investigation for their immunomodulatory effects: endothelial colony-forming cells-derived EVs were shown to protect kidneys from ischaemia-reperfusion injury[128,284]; human umbilical cord blood MSC-EVs intravenously injected, reduced the blood glucose levels and partially reversed insulin resistance in type II diabetes mellitus rat models[285]; human Wharton's jelly MSC-derived EVs were shown to induce regeneration of neuronal cells upon hypoxic ischemia-induced apoptosis in cell culture[286]; intravenously injected EVs derived from human cardiosphere-derived cells, rescued the dystrophic phenotype in Duchenne muscular dystrophy mouse models, leading to the ongoing clinical trial HOPE2[287].

The regenerative and immunomodulatory effects observed upon treatments with MSCs are known to depend on the tissue and donor sources[288], aspects that could be reflected on the therapeutic effectiveness of the secreted EVs and should be taken into consideration for future MSC-based therapeutic applications. In addition, the therapeutic capacities of MSC-derived EVs might, depending on the isolation method, vary among EV preparations with the possibility of co-purifying molecules that might act in synergy or in contrast to MSC-EVs.

### 2.8.2 EVs in Immunotherapy

In 1996, EVs derived from murine B lymphocytes were shown to carry MHC class II molecules able to induce an antigen specific T-cell response[124]. In parallel, DC derived exosomes (DEX) expressing MHC class I and II, pulsed with tumour specific peptides were found to induce tumour regression in mice, through activation of cytotoxic T-cells[125]. These studies paved the way towards the development of EV-based immunotherapies for cancer vaccination and infectious diseases. Up to now, four phase I/II clinical trials have been conducted using exosomes to elicit an immune response in patients with established cancers[29,289–291]. A study on non-small cell lung cancer patients, using DEX pulsed with different tumour peptides, showed a systemic rather than antigen-specific immune response and an increase in NK cells lytic activity[29]. In 2008, patients with advanced colorectal cancer were randomly treated with ascites-derived exosomes alone or combined with granulocyte-macrophage colony-stimulating factor (GM-CSF) and only the combined treatment induced tumour antigen-specific cytotoxic T cell response in two patients[289]. In both clinical trials, the DEX therapies were considered safe and well tolerated.

EVs are not exclusively secreted by eukaryotic cells, but also by bacteria, fungi and protozoa[292–294]. For instance, EVs of pathogenic non-eukaryotic origin have also been studied as vaccines. DEX pulsed with *Toxoplasma gondii* antigens, were shown to induce an immune response and protect mice against future *T. gondii* infections[295–297], whereas outer membrane vesicles derived from the bacteria *Neisseria meningitidis* were tested as vaccine to treat serogroup B meningococcal disease in adolescents and have entered the market[298].

These pre-clinical and clinical observations have proven the efficacy, tolerability and safety of EV-based vaccines to potentially combat both cancer and infections.

### 2.8.3 Bioengineered EVs

The above-mentioned natural features have been exploited to transform EVs into delivery vehicles for desired therapeutic molecules. Loading cargos of interest into EVs usually requires modifications of the EVs post-isolation either by co-incubation, sonication or electroporation (exogenous loading) or genetic manipulation of the parental cell to express molecules which are naturally incorporated into/onto EVs (endogenous loading) (**Figure 3**). These approaches have been used to impart EV specific characteristics for numerous purposes, described in detail hereafter.

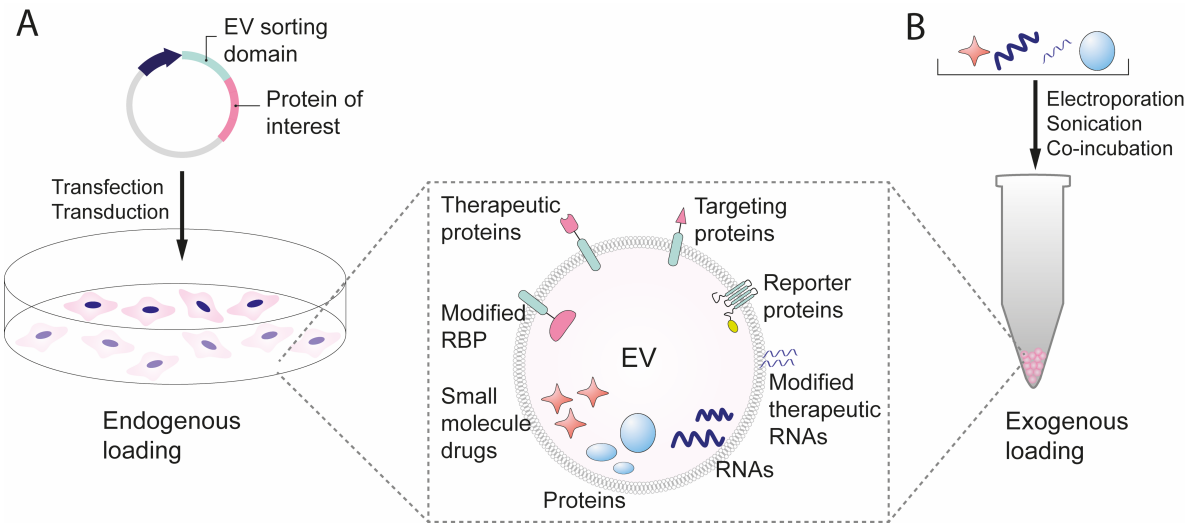


**Figure 3. EV loading strategies**. Loading EVs with cargos of interest can be achieved by endogenous (**A**) or exogenous loading (**B**). (**A**) Cells are transfected or transduced with plasmids expressing, for instance, a targeting or therapeutic protein fused to an EV sorting domain to enhance the loading into EVs (endogenous loading). (**B**) Once the endogenously engineered EVs are isolated, they can be further customised by loading small molecule drugs, modified therapeutic RNAs or proteins (exogenous loading). Similarly, engineered RNA Binding Proteins (RBPs) can also be employed to bind and load therapeutic RNAs into EVs. Figure inspired by[299].

### *2.8.3.1 Engineering EVs to improve biodistribution and tissue targeting*

In order to improve the circulation time and the tissue targeting, the surface of the EVs can be functionalized with different components. The hydrophilic PEG - known to increase the circulation of nanoparticles[300] - conjugated with anti-EGFR nanobodies, was co-incubated with mouse neuroblastoma cells (N2a)-derived EVs and intravenously injected. The authors reported an increment in circulation time and accumulation of PEGylated/EGFR EVs in EGFR-expressing tumour cells compared to unmodified EVs[301]. Another study reported an enhanced retention of fibroblast-like mesenchymal cells-derived EVs in the blood circulation upon intraperitoneal injection, due to the reduced phagocytosis of EVs carrying the 'do not eat me' CD47 molecule on their surface[208].

The functionalization of the EV surfaces with peptides or nanobodies to confer specific tissue tropism has also been explored and often performed in combination with therapeutic cargo loading to elicit site-specific effects in recipient cells. The first study of such kind endogenously loaded the EV associated protein Lamp2b fused to the neuron-specific rabies viral glycoprotein (RVG) peptide, into EVs. Post-isolation, the EVs were further electroporated with an siRNA directed towards *BACE1*, a target in Alzheimer's disease. Systemic injection of Lamp2b-RVG/siRNA loaded EVs resulted in an increased brain accumulation and a significant knockdown of *BACE1* at the mRNA and protein level[7]. A similar study employed another peptide (CP05) with high affinity for the extracellular loop of CD63. The delivery of EVs loaded with the CP05 peptide conjugated with a muscle targeting peptide and a dystrophin exon skipping phosphorodiamidate morpholino oligonucleotide, increased the restoration of dystrophin in muscles of dystrophic mice[263]. Nanobodies were also adopted to impart targeting

abilities to the EVs. The abundance of PS on the EV membrane was exploited to display antibody mimetics on the EV surface by fusing them to the C1C2 domain of lactadherin, known for its affinity to PS. Engineered anti-Her2 single chain variable fragments (scFVs) and anti-EGFR nanobodies, self-associated with EVs through the PS and once delivered, promoted the EV uptake by Her2+ human breast tumour xenograft mice[302] or EGFR+ cells[303], respectively.

#### *2.8.3.2 Engineering EVs to encapsulate therapeutic molecules*

EVs have been exploited as vehicles to deliver small molecules and drugs with low bioavailability. MSC-EVs conjugated with a targeting peptide via a biorthogonal click-chemistry and loaded with curcumin, , accumulated in the ischemic region of murine brains and suppressed local inflammation upon systemic administration[304]. These findings led to a phase I clinical trial, using curcumin loaded plant-derived EVs to treat colon cancer patients (NCT01294072). Bone marrow MSCs exposed to high levels of paclitaxel were shown to release EVs loaded with the chemotherapeutic drug which promoted the inhibition of tumour cell proliferation in culture[305]. Electroporation of doxorubicin into DC-EVs loaded with a specific αv integrin peptide (iRGD), displayed tumour tissue specificity in culture, further inhibiting the tumour growth in mice without any overt toxicity[306]. As previously mentioned, siRNAs have also been loaded into EVs either by electroporation or by hydrophobic modifications at the siRNA sequence level. EVs loaded with cholesterol modified siRNAs targeting Huntingtin mRNA, exhibit efficient silencing *in vitro*[307]. Intraperitoneal injection of fibroblast derived-EVs electroporated with KRAS siRNAs (iExosomes), dosed every other day, displayed tumour growth suppression and increased survival in various pancreatic ductal adenocarcinoma mouse models[208]. Based on this study, a clinical trial has been recently registered to treat metastatic cancer patients using iExosomes (NCT03608631).

#### *2.8.3.3 Designing EV platforms*

Beside the loading of therapeutic molecules, EVs have also been engineered for alternative purposes. For instance, in order to understand the biological mechanisms that drive the biogenesis, cell tropism and biodistribution of the vesicles, EVs are commonly customised to carry fluorescent or luminescent tags for *in vitro*[202,308] and *in vivo*[236,309,310] tracking. Further studies have described EV loading strategies that could potentially be adapted as universal platforms for different therapeutic applications. EXPLORs (exosomes for protein loading via optically reversible protein–protein interactions) is a platform that engineers EV associated proteins and soluble proteins of interest to be responsive to light stimuli. Upon light stimulation at the cellular level, the optogenetically modified proteins interact with each other during the EV biogenesis, leading to encapsulation of the soluble proteins into newly formed vesicles[311]. Kojima *et al.* developed a device named EXOsomal transfer into cells (EXOtic), composed of an exosome production booster, a neuronal targeting domain, an mRNA packaging and cytosol delivery tool. Once the producer cells, expressing the EXOtic device, were implanted in living mice, a delivery of the mRNA into neuronal cells resulted in decreased neuroinflammation and neurotoxicity in Parkinson's disease mouse models[223].

All these studies have demonstrated the immense potential of EVs as therapeutics and although still in the early stages, the exponential increase of studies unravelling the EV biology and optimizing EV production and manipulation, will bring these vesicles a step closer to clinical use in the near future.

# AIMS

The overall aim of this thesis is to address some of the challenging topics within the EV field towards therapeutic development. Firstly, it outlines the development of a novel EV isolation method, that allows for high EV recovery yields in a scalable and time-efficient manner. Secondly, includes an investigation on the interplay between the EV proteome and transcriptome, and thirdly encloses an extensive screening study on EV-associated proteins to advance EV engineering for potential therapeutic applications. The individual objectives relative to each paper are listed as follows:

## 3 Paper I

- To evaluate a novel liquid chromatography-based technique for EV purification, based on BE-SEC.
- To implement the BE-SEC method for isolation of EVs on a large-scale by adding a prior concentration and diafiltration step.
- To validate if the physicochemical properties of the EVs were compromised upon isolation.

## 4 Paper II

- To extensively characterise EVs endogenously labelled with an array of GFP-tagged EV marker proteins at the single molecule-single vesicle level.
- To compare the sorting efficiency of each protein into EVs using a set of diverse quantitative methodologies.

## 5 Paper III

- To investigate any differences in the proteome and small RNA transcriptome content of EVs derived from different cell lines.
- To explore, in particular, the relation between RNA/miRNA binding proteins and their vesicular RNA counterparts.

# METHODOLOGIES

## 1 Methodological Considerations

A detailed description of the methods employed in this thesis can be found in the respective papers. The following chapter has the sole purpose of giving a brief overview of the most important methods.

### 1.1 Cell Sources

In Paper I, to evaluate the TFF/BE-SEC method for EV purification, two different mouse cell lines were used: N2a (mouse neuroblastoma cells) and C2C12 (immortalized mouse myoblasts). For the cellular uptake, EVs derived from HEK293T (human embryonic kidney cells), stably expressing CD63-eGFP, were added to Huh7 (human hepato cellular carcinoma cells). In Paper II, to screen the different EV sorting proteins, HEK293T were used and transfected with the different protein expressing constructs. To characterize CD63-GFP labelled EVs in different cell lines, Huh7 and B16F10 (mouse melanoma cells) were employed. In Paper III, the RNA and protein content of EVs were investigated in HEK293T, RD4 (human skeletal muscle cells), N2a, C17.2 (immortalized mouse neural progenitor cells), C2C12 cell lines. The culturing conditions for each cell line aforementioned are described in the individual papers.

### 1.2 EV Enrichment Methods

Regardless of the isolation method employed in each paper, the initial steps were as follows: cell culture conditioned medium (CM) was collected and spun at 300 x *g* for 5 minutes, followed by 2000 x *g* for 10 minutes to remove floating cells and large cell debris. To enrich for small vesicles, the pre-cleared medium was filtrated with a 0.22 μm vacuum or syringe filter and subjected to different isolation steps. In Paper I, large volumes were diafiltrated and concentrated using KR2i TFF system (SpectrumLabs) with 100 or 300 kDa cut-off hollow fibers and subsequently run through the BE-SEC (HiScreen CaptoCore 700; GE Healthcare) column, connected to an ÄKTAprime plus (GE Healthcare). Small 0.22 μm-filtered media volumes were run directly through the BE-SEC column. After the BE-SEC column, the isolated material was concentrated using Amicon Ultra-15 10 kDa MWCO spin-filter (Millipore). In Paper II, in the experiments designed to screen the different sorting proteins, the processed CM was concentrated by UF with Amicon Ultra-15 100 kDa MWCO spin-filter (Millipore) to a final volume of 1 ml and loaded into qEVoriginal size exclusion columns (Izon Science). The vesicular fractions were collected according to the manufacturer' instructions and further concentrated with Amicon Ultra-0.5 10 kDa MWCO (Millipore) to a final volume of roughly 100 μl. For experiments where CD63-GFP was taken as candidate, pre-cleared CM was concentrated by UF and loaded onto a Superdex200 column (GE Healthcare) connected to an ÄKTA prime FPLC (GE Healthcare). Individual fractions were collected and further analysed. In Paper III, 0.22 μm -filtered CM was ultracentrifuged (UC) at ~120.000 x *g*, resuspended in

phosphate buffered saline (PBS), spun again at ~120.000 x *g* and the final EV pellet was reconstituted in roughly 100 µl of PBS. The UC was also adopted in Paper I and II.

## 1.3 EV Characterization

### 1.3.1 Nanoparticle Tracking Analysis

In all presented papers, the size distribution and concentration of particles were measured by Nanoparticle Tracking Analysis, using the NanoSight NS500 instrument (Malvern Ltd.) equipped with a 488 nm laser. Generally, five 30 seconds videos were recorded per sample and analysed with NTA 2.3 analytical software. The screen gain, detection threshold and minimum track length settings used for the analysis are described in the relative papers. Fluorescent particles were quantified on a constant flow to avoid bleaching of the fluorescent signal.

### 1.3.2 Western Blot

The EVs in all papers were evaluated for presence of EV markers by WB and compared with their donor cells. Equal number of particles were mixed with sample buffer (0.5 M ditiothreitol, 0.4 M sodium carbonate, 8% SDS and 10% glycerol) and heated at 65°C for 5 minutes. The cells were scraped, counted for viability with Trypan Blue, pelleted at 300 x *g* for 5 minutes and lysed with radioimmunoprecipitation assay (RIPA) buffer, kept on ice and vortexed every 5 minutes for half an hour. The cell samples were spun at 12.000 x *g* for 15 minutes at 4˚C, the supernatant was collected, mixed with the sample buffer and heated at 65˚C for 5 minutes. The mixture was then loaded onto a NuPAGE® Novex® 4-12% Bis-Tris Protein Gel (Invitrogen) and run at 120 V in NuPAGE® MES SDS running buffer (Invitrogen) for 2 h. The proteins on the gel were transferred to an iBlot nitrocellulose membrane (Invitrogen) using the iBlot system. Membranes were blocked with Odyssey blocking buffer (LI-COR) for 60 minutes at RT with gentle shaking. After blocking, the membrane was incubated overnight at 4˚C or 1 h at RT with primary antibody solution as described in each paper. The membrane was washed with PBST 1x (1x PBS with 0.1% Tween-20, Sigma Aldrich) 5 times every 5 minutes and incubated with the respective secondary antibodies (IRDye 800CW or 680LT, LI-COR). The membrane was washed with PBST 1x 5 times every 5 minutes followed by a final wash with 1 x PBS before imaging it on the Odyssey Infrared Imaging system using Image Studio Lite Version 5.2 (LI-COR).

### 1.3.3 Transmission and Cryo Electron Microscopy

In Paper I and III, TEM was employed to characterize the EVs. In both cases the EV samples were spotted on a glow-discharged formvar-carbon type B coated grid (Ted Pella Inc.) and stained with 2% uranyl acetate solution (Sigma-Aldrich). The grids were imaged with a FEI Tecnai 10 transmission electron microscope at an accelerating voltage of 100 kV. In Paper II, EVs were imaged via Cryo-EM. The samples were absorbed on glow-discharged holey carbon coated grids (Quantifoil) and vitrified into liquid ethane at -178˚C with a Vitrobot (FEI). The

images were recorded on a Philips CM200-FEG electron microscope (FEI) at an accelerating voltage of 200 kV, keeping the samples at -175˚C.

## 1.4 Single Vesicles Imaging

In Paper II, we screened the vesicular sorting capacity of several EV proteins. EVs derived from HEK293T cells transfected with different GFP-tagged EV domains, were characterized at the single vesicle level using diverse methods, as briefly described below.

### 1.4.1 Imaging Flow Cytometry

GFP-tagged EVs were analysed with the ImageStreamX MkII Instrument (Amnis), equipped with five lasers as described in detail in[88]. The samples were recorded using a 60x objective and a flow rate of 0.38 µl/min. GFP signals, brightfield and side scatter (SSC) were detected and the acquired data were analysed using Amnis IDEA software (v 6.2.64.0) and FlowJo).

### 1.4.2 Fluorescent Correlation Spectroscopy

EV samples were measured on a Clarina II Reader (Evotec Technologies) with 488 nm argon ion laser excitation, a 40x water immersion 1.15 N.A. objective (UAPO Olympus), 50 µm pinhole and a SPCM-AQR-13FC avalanche photodiode (Perkin-Elmer Optoelectronics). For each sample, several dilutions were made and measured in a 96-well glass bottom plate (Whatman) with 30 repetitive measurements of 10 seconds each. To determine the detergent sensitivity and quantification of GFP molecules per vesicle, the vesicles were disrupted with NP40s at 1% v/v (Nonidet P40 substitute, G-Biosciences). The fraction of intact vesicles was determined based on a two-component fit, setting the translational diffusion time of non-vesicular GFP to the values determined by a one-component fit in presence of 1 % NP40s.

### 1.4.3 Single Spotted Vesicles Imaging

EVs from single and double transfected HEK293T cells were spotted onto coverslips and imaged by confocal fluorescence microscopy (Zeiss LSM700) using 63x magnification or widefield with a 60x (API DeltaVision) on Photometrics CoolSNAP HQ2, interline transfer CCD. Vesicles were detected as light diffraction limited GFP or mCherry fluorescent spots of uniform size corresponding to the point spread function (PSF) of the microscope, confirming recovery of single vesicles. Widefield fluorescent images were deconvolved with the DeltaVision software fed with a measured 0.2 µm bead PSF for quantification. Co-localization was quantified based on overlap of the PSFs in the two fluorescent channels to derive the number of GFP, mCherry and GFP/mCherry double positive vesicles.

## 1.5 Flow Cytometry

Flow cytometry was employed in Paper I. Firstly, to evaluate whether the TFF/BE-SEC isolation method had an effect on the natural EV surface signature and secondly, to compare the EV uptake in recipient cells, upon different isolations.

### 1.5.1 EV Surface Protein Profiling

HEK293T derived EVs were isolated with UC and BE-SEC and diluted to the original particles concentration detected in the CM. EV staining with the MACSPlex Exosome kit, human (Miltenyi Biotec) was performed at 4˚C overnight according to the manufacturer's instructions. The MACSPlex Exosomes Kit is composed of capturing-beads coated with antibodies, directed against 37 different epitopes found on EVs. Once captured, the bead-bound EVs were stained with a mix of fluorophore-labelled antibodies directed towards CD9, CD81 and CD63 and analysed with a Cytoflex S flow cytometer (Beckman Coulter) with at least 10,000 recorded events per sample. Data were analysed with FlowJo software (version 10.0.7). The mean fluorescence values plotted in the graph were background corrected and normalized on CD63/81/9 mean signal intensity as previously described[66,312]. Negative values were excluded from the plot.

### 1.5.2 EV Uptake

For comparison, HEK293T:CD63-eGFP derived EVs were isolated by UC and TFF/BE-SEC. A fixed number of particles were added ($1x10^{10}$ and $5x10^{9}$ particles based on NTA scatter and fluorescence mode) to Huh-7 seeded the day before at a density of $7.5x10^{4}$ cells per well in a 24-well plate. Cells were incubated for 2 h at 37˚C, 5% CO2 atmosphere. After incubation, the cells were washed twice with PBS, collected, spun down at 300 x *g* for 5 minutes and resuspended in 100 µl of Dulbecco's PBS (Invitrogen), 1 mM EDTA and 2% FBS. Dead cells were excluded from analysis via 4',6-diamidino-2-phenylindole (DAPI) staining and doublets were excluded by forward/side scatter area *versus* height gating. Samples were kept on ice and measured with the Cytoflex S flow cytometer (Beckman Coulter). Data was analysed with the FlowJo software (version 10.0.7). Mean fluorescence intensity was normalized over the control/untreated cell sample (ΔMFI). Statistical significance was determined using GraphPad Prism (version 7.0b).

## 1.6 Next Generation Sequencing of Small RNAs

### 1.6.1 Sample Preparation and Sequencing

In Paper III, small RNA sequencing was performed on cells and their secreted vesicles. The RNA was isolated using Trizol or Trizol LS (Thermo Fisher Scientific) respectively, following the standard phenol-chloroform extraction protocol with a modified precipitation step that included the addition of 2 µl of PolyAcryl Carrier PC 152 polymer (Molecular Research Center Inc.) to enhance the recovery of small amounts of RNAs. The RNA concentrations were

measured using Qubit 2.0 Fluorometer with the Qubit RNA HS Assay Kit (Thermo Fisher Scientific), whereas the RNA integrity for the cell samples was verified on the Bioanalyzer RNA 6000 Pico Total RNA Kit (Agilent Technologies). Small RNA libraries were prepared with 250 ng of total RNA, using the NEBNext Multiplex Small RNA Library Prep for Illumina (NEB) kit according to the manufacturer's instructions. The barcoded samples were size selected on a 6 % Novex TBE PAGE gel (Thermo Fisher Scientific), the fragments corresponding to microRNA range were cut out and subjected to purification with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Thereafter, the products were quantified by using the KAPA Library Quantification Kit (Kapa Biosystems) and pooled at equimolar ratio. Two libraries (technical replicates) were generated in parallel, each eventually containing a pool of 12 barcoded samples. In both cases, the readymade libraries were checked on the High Sensitivity D1000 ScreenTape (Agilent Technologies) and further quantified using the KAPA Library Quantification Kit (Kapa Biosystems) to enable precise loading of the flow cell. The clusters were generated by using the cBot and sequenced one replicate per lane on either flow cells on the HiSeq2500 (Illumina Inc) with a 1x51 setup in RapidRun mode.

### 1.6.2 Data Analysis

In Paper III, raw sequencing reads were quality controlled by FastQC[313] analysis and subjected to adapter removal by Cutadapt/1.9.1[314]. All reads with an adapter and a length of 17-35 bases (filtering with BBMap release 35.40[315]) were subjected to subsequent mapping on the Ensembl 38.85 releases of the mouse and the human genome by using Bowtie1 (release 0.12.6)[316] in -v1 alignment mode and best alignment stratum reporting option. Annotation was performed in a stepwise manner with HTSeq (release 0.6.1)[317] in stranded mode by following a stepwise annotation procedure allowing the discrimination of 'small RNAs', followed by 'ribosomal RNA' and 'other RNAs'. Gene biotype classification followed the classification details in Vega Genome Browser release 68. The annotations of different RNA biotypes were retrieved from miRBase release 21[318], Ensembl 38.85[319], piRNAbank[320] and UCSC Table Browser hg38/mm10 entries[321]. For data visualization, MultiQC v1.3[322] and the online analysis software Morpheus (available from the Broad Institute; https://software.broadinstitute.org/morpheus) as well as Multiple Experiment Viewer (Version 4.9.0)[323] were used. Differential expression analysis of miRNAs was performed by using the R package DESeq2[324]. All statistical analyses (except for differential expression statistics) were performed using GraphPad Prism Version 7 (GraphPad Software).

## 1.7 Proteomic Analysis

The proteomic analysis of EVs in Paper II and Paper III exploited liquid chromatography-tandem mass spectrometry, as described previously[53]. The analysis covering Gene Ontology (GO) term enrichment and -overrepresentation was performed by using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) software[325]. In addition, the study included an in-depth analysis of the 'RNA binding' proteins (GO:0003723), obtained via the QuickGO

browser (http://www.ebi.ac.uk/QuickGO/) and Vesiclepedia[326] database. The list of 'miRNA related' proteins was created by manual curation of the 'RNA binding' proteins. All proteomic analysis was based on unique protein identifiers, thereby considering different protein isoforms.

In Paper II, we compared the proteomic content of CD63-GFP versus native EVs. The samples were analysed by SDS-PAGE on a NuPAGE 4-12 % (Life Technologies) gel and stained with a Colloidal Coomassie stain (Sigma). Sixteen equal sized slices were excised from each of the gel lanes. In-gel digestion and subsequent identification by liquid chromatography coupled with tandem mass spectrometry was performed as previously described[327], with the exception that a mix of Trypsin and Endopeptidase Lys-C (Promega) was used instead of trypsin alone. Database searches were done with Mascot (version 2.4, Matrix Science) against the UniProt database (release of April 2013) concatenated with a reversed version and supplemented with known contaminants (such as trypsin, BSA and commonly used tags). Protein identifications were validated and summarized in Scaffold (version 4.0.3, Proteome Software Inc.), setting the protein identification threshold at a 1 % false discovery rate (FDR) in the reversed database. At these settings, peptide FDR was 0.05 %. Keratin contaminants were removed and are listed separately. Trypsin and Lys-C were also removed from the list. Total spectral count is provided as a semi-quantitative measure, as well as the number of unique peptides for each protein.

# RESULTS AND DISCUSSION

## 2 Paper I

EVs are isolated from cell cultures or biological fluids but due to their small size, their purification is a challenging endeavour. UC is still one of the most utilised EV isolation methods in the field[51], although it has its limitations[54,328]. For this reason, many alternative techniques have been described to date[70,91,329]. In this work, we evaluated a novel EV purification technique that combines size exclusion with bind-elute chromatography.

Firstly, we assessed the feasibility of isolating EVs with the BE-SEC column, using pre-cleared CM from two mouse cell lines (N2a and C2C12). The eluted volume was further concentrated and the retentate was analysed for the presence of vesicles. At first, similar particle size distribution and concentration were observed across six different replicates and once compared to the input material, a particle recovery rate ranging from 70 to 80 % was achieved. The particles were further characterized by immunoblotting and the classical vesicular markers Alix, CD81 and Tsg101 were detected among different replicates, denoting the presence of EVs in the BE-SEC eluate. In addition, the size and morphology of the vesicles were evaluated by TEM, showing non-fused intact vesicles of expected sizes. All these data suggested a successful EV isolation using the BE-SEC column.

For the purification of EVs in clinal settings, scalability is one of the requirements. Therefore, we coupled the BE-SEC column with a Tangential Flow Filtration (TFF) device, combined called TFF/BE-SEC, to process larger volumes of CM, by concentrating and diafiltrating the sample. The TFF system consists of hollow fibres through which the sample flows tangentially along the semipermeable membrane decorated with pores of different sizes. Herein, we tested 100 and 300 kDa hollow fibre cut-offs prior to the BE-SEC column isolation. The EV characterization of the isolated material, showed analogous results to what described above in terms of markers expression and morphology. As expected, we detected more proteins being retained in the 100 kDa cut-off TFF filters compared to the 300 kDa ones, and considerably less impurities were observed upon BE-SEC isolation, regardless of the TFF cut-off filter used. Furthermore, the content of both isolated samples (TFF or TFF/BE-SEC) was subjected to analytical SEC, enabling the size fractionation and discrimination between vesicular and non-vesicular material. Both protein and RNA concentrations were measured in each eluted fraction and upon BE-SEC isolation, the non-vesicular components were undetectable indicating a successful removal of the extra-vesicular material.

As previously reported, the EV surface protein composition determines the EV biodistribution[330] and cellular uptake[196,331], therefore the surface protein profile of EVs isolated by UC and TFF/BE-SEC was assessed by flow cytometry multiplex beads-based assay[66,67]. The analysis revealed that the surface protein profile of both isolated EV samples were similar, reflecting the composition of vesicles contained in the unprocessed starting material. Hence, based on this assay, the TFF/BE-SEC purification method did not seem to alter the EV surface protein composition. Any changes in the cell uptake were also investigated, employing

HEK293T:CD63-GFP EVs isolated by UC and TFF/BE-SEC. The CD63-GFP EVs internalization in Huh7 cells, evaluated by flow cytometry, showed higher MFI values for the UC isolated samples, reflecting a 20% higher amount of GFP positive particles detected by the NTA. These discrepancies were evened out once the same number of GFP positive particles were given to cells. No other significant differences were detected upon isolation between the methods.

In conclusion, we describe an isolation method that combines size exclusion with tangential flow filtration and can perform EV purification in a scalable, time-efficient fashion providing high vesicular yields.

# 3 Paper II

Due to the EV heterogeneity and the recent discoveries describing the existence of various vesicle subpopulations, there is a pressing need for the development of straightforward methodologies to quantitatively characterise extracellular vesicles at the single vesicle – single molecule level. In addition, linking vesicular doses to the molecular concentrations of EV cargo is a question of utmost importance for the development of EV based therapeutics and understanding the basic vesicle biology alike. Hence, in this study, we provide a systematic comparison of several EV sorting domains and describe a set of relatively straightforward methodologies for single molecule – single vesicle quantification, that could represent a valuable resource for researchers in the EV field.

To start with, we characterized EVs derived from HEK293T transfected with CD63-GFP (N-terminus), one of the first tetraspanin proteins to be described in exosomes[110]. EVs were fractionated by UF-SEC[91], individual fractions were pooled and analysed by immunoblotting. The EV markers Alix and Tsg101 eluted in the expected EV fractions, similarly to Lamp2b that in addition showed a broader elution profile, indicating the possibility of being associated with a different vesicle population. CD63-GFP fusion protein also eluted in the EV fractions, but a truncated GFP was observed to elute in later fractions, according to WB and FCS. We then set up a workflow to characterize single GFP labelled vesicles via FCS. To resolve and quantify the association and the number of CD63-GFP molecules per EV, we tested different conditions to disrupt the vesicles and allow protein solubilization. Among all the different chemical and physical treatments, NP40s showed, at the lowest concentration, the highest levels of disruption resulting in a homogeneous population of molecules with brightness similar to free GFP. Based on these observations, we were able to quantify 10 to 30 GFP molecules per single vesicle and determine that the GFP eluting in later fraction was consistent in size and brightness to monomeric GFP, hence mainly non-vesicular.

Next, we tested different transmembrane, membrane-associated, soluble proteins and their potential EV sorting capacity. For this scope, we screened 12 different proteins spanning from EV markers[90,97,118,332] to proteins involved in the EV biogenesis[333], proteins previously

engineered for tissue-targeting[334] or sorting moieties[335–337] and tags used to anchor soluble proteins to the membrane[338–340]. All protein coding sequences were fused to GFP as readout and untagged GFP was used as reference. The constructs were then transiently transfected into HEK293T cells using polyethylenimine and 48h later EVs were isolated by a simplified SEC protocol (UF-qEV) consisting of a 100 kDa ultrafiltration step followed by a qEV size exclusion column to separate the vesicular and non-vesicular components. The size and particle concentration of the isolated EVs were analysed by NTA and the expression of the EV markers and GFP was validated by immunoblotting, revealing a truncated form of GFP in most of the samples as previously observed for CD63-GFP. Most of the transmembrane proteins were detected in EVs with the exception for CD63 (2$^{nd}$ loop), whereas for the soluble and membrane associated proteins the highest levels were observed for Syntenin, SIMPLE and Myristoylation tag. These data were additionally confirmed by single vesicles confocal imaging and imaging flow cytometry[88]. The expression was also compared to the donor cells: when immunoprobed for GFP the predicted molecular weight for each protein was confirmed by WB and the transfection efficiency ranged from 20 to 40% according to flow cytometry.

As with CD63-GFP EVs, we characterized all the differently tagged EVs with FCS. Based on this method, the GFP-tagged tetraspanins exhibited 25-35 fluorescent molecules per vesicle on average, with CD63 (N) showing the highest number per vesicle and CD63 (2$^{nd}$ loop) the lowest, indicating how the sorting of CD63 is sensitive to the GFP tagging in specific regions. The Myr tag resulted in efficient GFP loading, with similar number of molecules per vesicles to the tetraspanins. The other tested proteins such as Lamp2b, Syndecan, MFGE8 (C1C2), Alix, SIMPLE and Flotillin-2, displayed very dim and little fluorescent molecules per particle.

Based on recent papers addressing the existence of different EV subpopulations with unique content signatures[32,90,341], we investigated the vesicular heterogeneity in relation to sorting different proteins into EVs by exploiting the GFP-tagged proteins in combination with mCherry-CD63. All GFP-tagged proteins previously adopted were transiently co-transfected with mCherry-CD63 in HEK293T cells, the secreted EVs were isolated and characterized as previously described. To evaluate the GFP and mCherry co-localization on dual labelled vesicles, we imaged EVs spotted on coverslips using confocal and widefield fluorescent microscopy and quantify the number of GFP, mCherry or double positive dots. For most of the tested construct, we observed that 50% of the GFP-vesicles carried mCherry-CD63, regardless of the GFP-protein expression levels in the EVs, exception for Lamp2b that was predominantly detected in EVs negative for mCherry-CD63, suggesting a potential diverging vesicular trafficking biogenesis.

In summary, we describe a set of analytical techniques used to quantify and characterize single vesicles at the molecular level. Moreover, we adopt these methods to provide a systematic comparison of several EV associated proteins and evaluated their EV sorting capability with the potential to replace the fluorescent tag with relevant therapeutic cargos.

# 4 Paper III

The innate biological effects mediated by EVs are attributed to their cargo, in particular to proteins and RNAs, that are vastly adopted to engineer EVs for therapeutic approaches and therefore require thorough investigation. In this paper, we examined the small RNA and protein content of EVs across different human and mouse cell lines.

At first, small RNA sequencing on UC-purified EVs and their source cells (human HEK293T, RD4 and mouse C2C12, N2a and C17.2) was performed. The libraries and the data analysis were size-selected and restricted to sequences of 17-35 nucleotides in length and indeed, 80% of the cellular RNA was represented by 'small RNA' sequences, of which 73-93% were miRNAs. On the other hand, EVs had considerably less miRNAs and more piwi-like RNA (piRNA) sequences than the cells of origin and was observed regardless of the cell type. Hierarchical clustering analysis of the miRNAs, rather clustered EVs with their parental cells as opposed to EVs-derived from other cell sources. Similarly, the number and relative expression of miRNA sequences in EVs were correlating with the cells of origin; conversely, piRNA sequences did not show clear correlation between the expression level in cells and vesicles. Considering this, together with the lack of evidence of typical piRNA features (e.g. 27-35 nucleotides in length, 5' uracil bias), these RNA sequences are more likely to be derived from piRNA loci rather than genuine piRNAs. Even though the majority of the cellular sequences were represented by 'small RNAs', the EV 'small RNA' content was highly variable across cell lines, with an average of ~ 22% sequences derived from 'small RNA' loci whereas the 36-94% of all annotations in EVs were mapping to rRNA loci. A large number of tRNA and Y RNA sequences were found in both cells and EVs, supporting previous EV 'small RNA' sequencing studies[145,342–344].

Next, we explored the correlation between the transcriptome and proteome, with a focus on the RNA-binding proteins, in HEK293T and C2C12 EVs given their diverse 'small RNA' content. In both proteomes, ~2000 proteins were detected, of which ~200 covered the bulk content (75%) of both proteomes, similarly to what was observed in the transcriptomics data. In line with the sequencing results, where ribosomal, coding and tRNA fragments were highly abundant, high levels of rRNA-, poly(A)- and tRNA binding proteins were identified in both EV proteomes. The GO analysis revealed that ~20-30% of the proteins identified in the EVs were classified as 'RNA binding' (GO:003723), correlating to what reported (21%) in the Vesiclepedia database[326]; of these 'RNA binding' proteins, the majority was represented by poly(A)-, rRNA-, double- and single- stranded RNA binding proteins and translational related protein sets. To further understand which classes of RNA-binding proteins are represented in our data set and how they could be related to the 'small RNA' transcriptome, we generated a custom curated GO list of the 'miRNA associated' proteins. Based on this GO annotation, only ~1% of the HEK293T and C2C12 EV proteins were relevant to the miRNAs molecular and biological processes. Even though C2C12 contained higher miRNA levels than HEK293T EVs, no differences related to the miRNA proteome were observed, therefore we could not link the presence of certain proteins to an active sorting of miRNAs into EVs. Similarly, no 'piRNA

binding' proteins were detected in the EV proteome, corroborating the hypothesis that the sequences annotated as piRNAs were rather reads mapping to the piRNA loci.

In summary, we explored the link between the EV transcriptome and proteome across different human and mouse cell types. Moreover, this study paves the way for future work that could unravel the biological mechanisms underlying the active RNA sorting into EVs and exploit the obtained data sets to engineer vesicles for RNA-based therapeutic applications.

# CONCLUSIONS

In the last decades, much progress has been made in understanding the biology of EVs. Owing to their ability of transferring bioactive molecules across cells, EVs have an impact on several physiological and pathological processes such as stem cell plasticity, neuronal communication, inflammation, tumorigenesis and neurodegenerative diseases. Recently, the possibility of harnessing the EV content to monitor the progression or state of certain diseases, has proven the feasibility of using EVs as liquid biomarkers. Moreover, exploiting EVs as carriers to deliver defined therapeutic molecules *in vitro* and *in vivo* have shown promising results. Analogously, several ongoing pre-clinical and clinical trials have proven the safety and tolerability of these vesicles. Therefore, EVs hold great potential as prognostic, diagnostic and therapeutic vectors. There are, however, challenges that hinder the clinical use of EVs on a large scale. Most of these hurdles are technical obstacles such as culturing conditions, optimal isolation, improved manipulation and proper storage, and the lack of sensitive methodologies for tracking the fate of single EVs within cells and organisms, in studying their biological function.

Efforts have been made to advance the field and, in this thesis, we have also tackled some of the current challenges. We addressed standing gaps in the large-scale isolation of EVs; in EV bioengineering, quantifying the molecular cargo at a single vesicle level, and in dissecting the molecular content of EVs across several cell lines. Even though the EV field is still in its infancy and much more as yet to be discovered, we are confident that these small progresses will advance these vesicles one step closer to their large-scale clinical applications.

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