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PURIFICATION AND BIODISTRIBUTION OF EXTRACELLULAR VESICLES

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Purification and Biodistribution of Extracellular Vesicles

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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For my wonderful wife

ABSTRACT

Extracellular vesicles (EVs) are nano-sized vesicles that contain bioactive lipids, RNAs and proteins, which can be transferred to recipient cells. EVs are important for physiological as well as pathological processes, such as coagulation and immune homeostasis, aiding cancer metastasis and spread of infectious diseases. Owing to their relatively small size the purification of EVs is a challenge, hence we have established and optimised workflows consisting of ultrafiltration with subsequent size exclusion liquid chromatography (UF-LC)(Paper I) and bind-elute combined with size exclusion (BE-SEC) columns (Paper III) for EV purification.

UF-LC allowed for purification of biophysically intact EVs with better yield and purity compared to ultracentrifugation (UC), which is the gold standard purification method in the field. The biodistribution of UF-LC EVs was different compared to vesicles isolated using UC, despite having highly similar protein composition according to proteomics analysis. We found that UF-LC vesicles accumulated less in lung, possibly owing to their higher integrity. Indeed, fluorescence correlation spectroscopy and transmission electron microscopy indicated that the high gravitational forces in UC lead to aggregation and disruption of the vesicles. The BE-SEC method is a similar method to UF-LC, however protein impurities less than 700 kDa in size are bound in the interior of the beads, thus improving simple size-based exclusion. The BE-SEC method is scalable, produces samples with better purity than UC, displaying yields exceeding 70% and demonstrates a good reproducibility between samples. Moreover, vesicles purified by BE-SEC display the same EV surface markers as UC purified EVs, and CD63-eGFP positive vesicles are taken up in recipient cells to the same extent. In summary, the BE-SEC method is a reproducible and fast alternative to UF-LC for large media volumes.

Reliable purification methods are important for the implementation of therapeutically active EVs, however knowledge regarding their eventual organotropism and biodistribution is equally important. Thus, in article II we evaluated the biodistribution of EVs specifically labeled with a near-infrared dye. The main sites of accumulation of exogenously injected EVs were liver, spleen and lungs. Biodistribution profile of EVs depended strongly on injection route, and to certain extent, on EV cell type source, as dendritic cell derived EVs exhibited a more pronounced uptake in spleen compared to the other cell sources tested. We further showed that small alterations of EV surface proteins could significantly affect biodistribution as well, since EVs equipped with a brain targeting peptide on their surface increased the uptake of targeted EVs in brain. This study highlights that the biodistribution of EVs follows other nano-sized particles with uptake mainly in liver. Administration route, cell source and a targeting peptide influence the distribution, however the overall distribution is unaltered with the highest signal originating from liver.

To summarise, this thesis has resulted in improvements of the EV field by systematically enhancing EV isolation workflows to achieve greater sample purity and at the same time preserving EV biophysical characteristics. Furthermore, it has laid groundwork for studying *in vivo* effects of exogenous vesicles. Both these aspects are particularly important for understanding EV biology more clearly and with increased detail.

LIST OF SCIENTIFIC PAPERS

I. **Ultrafiltration with size-exclusion liquid chromatography for high yield isolation of extracellular vesicles preserving intact biophysical and functional properties**
Joel Z. Nordin*, Yi Lee*, Pieter Vader, Imre Mäger, Henrik J. Johansson, Wolf Heusermann, Oscar P.B. Wiklander, Mattias Hällbrink, Yiqi Seow, Jarred J. Bultema, Jonathan Gilthorpe, Tim Davies, Paul J. Fairchild, Susanne Gabrielsson, Nicole C. Meisner-Kober, Janne Lehtiö, C.I. Edvard Smith, Matthew J.A. Wood, M.D., Samir EL Andaloussi
Nanomedicine: Nanotechnology, Biology, and Medicine, 2015, 11, 879–883

II. **Extracellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting**
Oscar P. B. Wiklander*, **Joel Z. Nordin***, Aisling O'Loughlin, Ylva Gustafsson, Giulia Corso, Imre Ma¨ger, Pieter Vader, Yi Lee, Helena Sork, Yiqi Seow, Nina Heldring, Lydia Alvarez-Erviti, CI Edvard Smith, Katarina Le Blanc, Paolo Macchiarini, Philipp Jungebluth, Matthew J. A. Wood and Samir EL Andaloussi
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III. **Fast and reproducible purification of extracellular vesicles using combined size exclusion and ion exchange chromatography**
Giulia Corso, **Joel Z. Nordin,** Imre Mäger, Yi Lee, André Görgens, Jarred Bultema, Bernd Giebel, Matthew J.A. Wood and Samir EL Andaloussi
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***** These authors contributed equally to the manuscripts

CONTENTS

LIST OF ABBREVIATIONS

A4F	Asymmetrical-flow field-flow fractionation
AFM	Atomic force microscopy
ARF6	Adenosine diphosphate-ribosylation factor 6
BE-SEC	Bind-elute with size exclusion chromatography
CCR5	C-C chemokine receptor 5
DC	Dendritic cell
DiR	1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
EGFR	Epidermal growth factor receptor
ESCRT	Endosomal sorting complexes required for the transport
EV	Extracellular vesicle
FCS	Fluorescence correlation spectroscopy
FBS	Fetal bovine serum
g/ml	Gram/milliliter
GFP	Green fluorescent protein
HSPG	Heparan sulfate proteoglycans
ILV	Intraluminal vesicle
IP	Intraperitoneal
ISEV	International Society for Extracellular Vesicles
IV	Intravenous
IVIS	In Vivo Imaging System
LC	Liquid chromatography
LN	Lymph nodes
MFD	Microfluidic device
MFGE8	Milk fat globule-EGF factor 8
MHC-II	Major histocompatibility complex II
MPS	Mononuclear phagocytic system
mRNA	Messenger RNA
miRNA	MicroRNA
MSC	Mesenchymal stromal cell
MV	Microvesicle
MVB	Multivesicular body
N2a	Neuro2a
nm	Nanometre
NTA	Nanoparticle tracking analysis
qPCR	Qualitative polymerase chain reaction
PDCD6IP	Programmed cell death 6 interacting protein
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PS	Phosphatidylserine
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SC	Subcutaneous
SEC	Size exclusion chromatography
TEM	Transmission electron microscopy

TFF	Tangential flow filtration
TRPS	Tunable resistive pulse sensing
TSG101	Tumour susceptibility gene 101 protein
UC	Ultracentrifugation
UF	Ultrafiltration
UF-LC	Ultrafiltration with subsequent size exclusion LC
WB	Western blot

1 INTRODUCTION

1.1 BRIEF HISTORY OF THE EV FIELD

The field of extracellular vesicles (EVs) goes back as far as to 1946, when it was discovered that high-speed centrifugation of plasma prolonged the clotting time of the supernatant. When the pellet was re-introduced to the supernatant, the clotting time was normalised (1), thus, suggesting that cell-free plasma contains a "clotting factor" larger than most proteins. This "clotting factor" was determined to be small vesicles by electron microscopy in 1967 and they were referred to as "thrombocyte dust" (2). In the following years, several vesicle-related articles were published, in other biological systems; these included reports regarding vesicles involved in bone calcification (3), particles in fetal bovine serum (4), cellular fragments from cancer cells (5, 6), vesicles from rectal adenoma microvillus (7) and vesicles in seminal fluid (8). In 1983, two articles indicated that EVs had a real biological significance when two independent groups demonstrated that EVs were responsible for the shedding of the transferrin receptor in maturing reticulocytes as well as proving that the vesicles originated from multivesicular bodies (MVBs) (9, 10). In the 1990s, the field of EVs advanced further when it was discovered that EVs play a role in immune regulation and could elicit a T-cell response (11). However, it was not until 15 years later that the field gained increasing attention when three different groups showed that EVs carry ribonucleic acids (RNA) and proteins and that these biological cargoes could be transferred to recipient cells in various model systems (12-14). Today, EVs are recognised as important intercellular messengers in both physiology and pathology (15). The field has expanded rapidly the last decade, which is evident by the exponentially growing number of publications annually on the NCBI Pubmed website (Fig. 1).

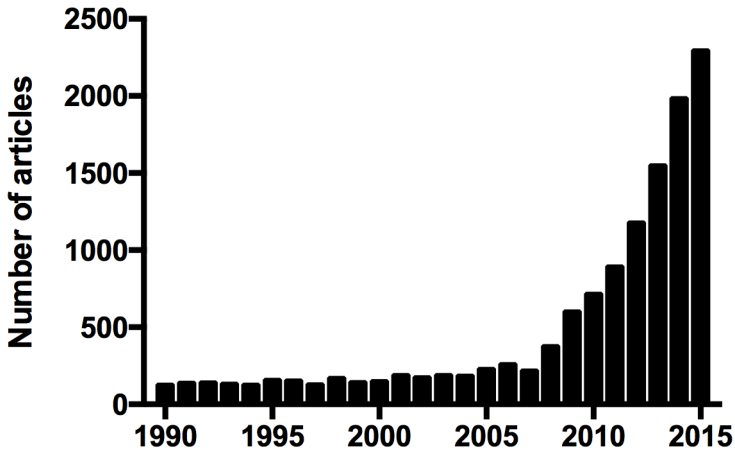

Figure 1. Total number of publications retrieved on PubMed.org (as of 12th of July 2016) with the search terms "exosomes", "micro-vesicle" and "extracellular vesicle" combined, for the time period 1990-2015.

1.2 CLASSIFICATION OF EVS

The nomenclature in the EV field has been under constant debate. EVs have been named in innumerable ways during the maturation of the field. Previously, it was common practise to name vesicles based on their originating cell type, such as prostasome (8) (prostate cell EVs), dexasome (16) (dendritic cell (DC) EVs), matrix vesicles (17) (cartilage and bone EVs) and synaptic vesicles (18) (neuronal EVs). However, others have classified EVs based on their biogenesis and origin within the cell into three groups (19); exosomes, microvesicles (MVs) (also referred to as shedding vesicles, shedding microvesicles, or microparticles) and apoptotic bodies (also named apoptotic blebs, or apoptotic vesicles). The main characteristics for each group are described in Table 1. However, the discussion regarding the classification is still ongoing where researchers have advocated the inclusion of more sub-groupings such as ectosomes, membrane particles and exosome-like vesicles (20). However, any such

classification is likely an oversimplification as recent evidence clearly suggest high heterogeneity even within one vesicle type (21).

Vesicle Type	Size	Density (g/mL)	Morphology (in TEM)	Origin	Markers (enriched)
Exosomes	40-120 nm	1.13–1.19 (22)	Cup-shaped (22)	Endosomes	Tetraspanins, PDCD6IP, MFGE8 etc.*
Microvesicles	50-1000 nm	1.03-1.08 (23)	Cup-shaped (23)	Plasma membrane	Integrins, CD40 ligand*
Apoptotic bodies	500-2000 nm	1.16–1.28 (24)	Heterogeneous (25)	Plasma membrane, endoplasmic reticulum	Phosphatidylserine, DNA and histones*

Table 1. Characteristics of exosomes, microvesicles and apoptotic bodies. * Not specific for the particular vesicle type. g/mL: gram/millilitre, TEM: Transmission electron microscopy, PDCD6IP: Programmed cell death 6 interacting protein, MFGE8: Milk fat globule-EGF factor 8, DNA: Deoxyribonucleic acid.

Exosomes are generally 40-120 nanometres (nm) in diameter, derived from the late endosomal pathway within the cytoplasm and are the most well-characterised of the three subtypes (15). MVs bud directly from the plasma membrane and are 50-1000 nm in size (15) whereas apoptotic bodies are released from apoptotic cells and are more heterogeneous in size distribution (15). Recently, there is increasing number of studies that suggest subpopulations of vesicles with different biological properties and phenotypes within each of these subgroups (21), further adding to the complexity of vesicle research. In this thesis, the term EV will be used to describe all cell-derived vesicles in general, with the exception of apoptotic bodies. In certain sections and depending on the context, the other two vesicle subgroups (exosomes and MVs) may be specifically defined.

1.3 METHODS FOR EV CHARACTERISATION

As can be seen from Table 1, vesicles can be classified based on their size, morphology, density and protein composition. To date however, there is still no exclusive marker for differentiating between exosomes or MVs, even though they have different biogenesis pathways (26, 27). Our current methods applicable for cellular work are limited in determining the exact vesicle composition within a biological sample, which is likely to be highly heterogeneous and stochastic to a certain extent, given the thousands of individual proteins detected in an EV sample and the limited EV surface area and volume.

1.3.1 Size characterisation

One important issue that restricts classification of EVs based on their physicochemical parameters is that these nano-sized vesicles are below the detection threshold for normal light microscopies (28), thus more specialised alternatives, such as super resolution microscopy, transmission electron microscopy (TEM) (11), atomic force microscopy (AFM) (29) or similar apparatuses are required. Although modern versions of flow cytometers can now detect vesicles down to around 150 nm in diameter, the vast majority of exosomes is smaller and therefore excluded (30). New imaging flow cytometers can hopefully detect sub-100 nm vesicles, however the technique needs to be further investigated before any clear conclusion can be made (personal communication with Dr. A. Görgens). Nanoparticle tracking analysis

(NTA) and tunable resistive pulse sensing (TRPS), are examples of newer developments tailored for nanoparticle detection, and can be used to assess global particle size distribution and concentration in a sample (31, 32), where the size distribution of EV samples often resembles a Gaussian profile. Unfortunately, both techniques have a common flaw where EVs, protein complexes and/or lipid particles may all mistakenly be interpreted as being vesicles. Additionally, operators may manually set thresholds and post-acquisition settings as desired and subsequently affect the reproducibility and reliability of NTA results across research groups. Thus, these technologies in their current stage should not be used for absolute quantitation, but rather as relative measurements in a given experimental setting or for describing EV batch-to-batch variability.

1.3.2 Protein evaluation

To assess the protein composition of EVs, western blot (WB), antibody coated beads for flow cytometry as well as mass spectrometry-based proteomics are commonly used (33, 34). However, these techniques measure the protein composition of the whole sample and not single vesicles. Furthermore, depending on the pre-processing procedure, it is hard to be certain whether the identified protein is indeed originating from the EVs or from co-precipitating protein complexes. To further study the protein composition, immuno-EM can be utilised, which can visualise proteins on the EV surface by immuno-gold secondary antibody staining. Fluorescence correlation spectroscopy (FCS) and similar specialised equipment can also be utilised to analyse the EVs on a single vesicle level. The downside with FCS is that it can only analyse fluorescently labelled EVs, hence either a dye or a genetically engineered construct has to be introduced, which limits the analysis to a single or only a handful of proteins, similarly to the immune-EM based technologies.

1.3.3 EV density

Another EV characteristic is its buoyant density that can be measured by density gradient centrifugation, using e.g. sucrose or Optiprep™ gradients (20). However, EV density measurements are complicated because spin time and loading principle can significantly affect the measurement. Additionally, the sucrose gradient is of a hyperosmolar nature (35), which can further influence the results by changing water content of vesicles due to osmotic pressure. Current evidence nevertheless suggests that EVs can be purified from contaminants based on their differential density and that the density of EV subtypes can be different too (21), which is important for certain applications.

1.3.4 Evaluation of RNA content

The deoxyribonucleic acid (DNA) and RNA content of the vesicles is another commonly investigated parameter. However extracellular RNA does not only exist in EVs, it can also be found as free RNA and bound to proteins or lipoprotein particles (36-38), hence it is important to ensure that the purified RNA actually stems from EVs before any analysis is undertaken. Thereafter the RNA content can be analysed with several different methods such as qualitative polymerase chain reaction (qPCR), digital PCR, northern blotting, next generation sequencing, or simply by fluorescent RNA specific dyes. Depending on the method chosen the quality of the obtained data and detection sensitivity can differ. Additionally, depending on the extraction method, certain RNA species can be purified from EV samples more efficiently than others, possibly introducing bias to RNA analysis and posing challenges for data normalization (39). Therefore, similarly to the analysis of EV proteins, it is often preferred to analyse changes of a given RNA species within EVs at different conditions rather than comparing absolute RNA copy numbers which is more sensitive to sample processing biases.

1.3.5 Pre-analytical considerations

Characterisation of EVs in biofluids is even more complicated than in cell culture supernatant because there are additional aspects to consider. All the characterisation methods described above are additionally influenced by pre-analytical methods, such as venepuncture techniques, buffers and anticoagulants used when extracting EVs from plasma or serum, and specifics related to biofluid type (e.g. urine, breast milk or saliva). These are similar issues to the cell culture where culturing conditions can significantly affect the characteristics of isolated EVs (29, 40).

The existing challenges in EV characterisation, as described above, clearly emphasise that in order to understand the composition of EVs as artefact-free as possible, it is critical to use sufficiently reliable purification methods in the initial sample-processing step. This is also recognised by the International Society for Extracellular Vesicles (ISEV) who released a statement addressing the minimal characterisation requirements for EV research. ISEV highly recommends the use of several different techniques when characterising vesicles, since there is no single method that can reliably characterise any given vesicle sample (41). The field, nevertheless, has advanced considerably regarding the methods available for the characterisation of vesicles, however, it remains extremely difficult to examine vesicles on a single vesicle level and it is still unclear how storage conditions and the use of different buffers may affect the biophysical and biological properties of vesicles. In the coming years, the field will hopefully come to a consensus on the fundamentals of EV research such as buffers, storage conditions and more optimisation on current purification methods.

1.4 EV PURIFICATION METHODS

The purification of EVs has always been a great challenge due to their small size, biochemical properties and particularly the complexity of the surrounding fluid. Importantly, one needs to pay attention to the selected method for EV purification as it can significantly affect the downstream biological results. Whether it is biological fluids or cell culture supernatants, one needs to be aware that these are highly heterogeneous fluids containing proteins, non-exosomal RNA, dead cells and cell debris as well as lipoprotein particles, and possibly other additives in addition to EVs. Blood/plasma/serum is particularly difficult to study due to the high viscosity, high abundance of ‘sticky’ proteins, such as albumin, and lipoprotein particles and certain protein multimers, such as von Willebrand factor, which is in the same size range as EVs (29).

1.4.1 Ultracentrifugation

The current gold standard for EV purification is differential centrifugation for the sedimentation of vesicles in solution (42). As the high-speed centrifugal forces can pellet dense and large particles, a series of lower-speed centrifugation steps with increasing speed/centrifugal force are undertaken to initially separate vesicles of different sizes/densities. Pelleting efficiency is determined by several factors including, size, density and shape of the vesicle, temperature, viscosity and volume of the medium, and whether a fixed or swing-out rotor is used (43). The low speed spins normally include a first 300-500*g* spin to get rid of cells and large cell debris, followed by a 1500-2000*g* spin to get rid of smaller cell debris. After the 2000*g* spin the medium can either be filtered through a 0.2 μm filter or spun at 10 000-20 000*g* to separate out vesicles larger than 100-200 nm in size (usually referred to as MVs). The last step is the ultracentrifugation (UC) spin at 100 000-200 000*g* to pellet vesicles under 100-200 nm (usually referred to as exosomes). The latter step can be repeated as a washing step to achieve higher EV purity (42, 44).

Recently, some apprehension has emerged concerning the purity, yield, aggregation, intactness and functionality of the vesicles after UC purification (45-48). UC has been shown to pellet EVs as well as contaminating proteins and low-density lipoprotein particles. Furthermore, aggregates can be present that reduce the therapeutic effectiveness of the vesicles or be misleading when studying the active component of the preparation (45, 48, 49). Adding a sucrose gradient purification step reduces protein contamination considerably (50), however the sample remains contaminated by high density lipoprotein particles present and the vesicles are not as functional after sucrose gradient purification (51, 52). It is also problematic to scale up the UC process, since most UC rotors are limited to handle up to 400 mL solution in one run (45). Finally, the technique is highly operator dependent.

1.4.2 Alternative purification methods

Consequently, many alternative purification methods have emerged, such as commercially available precipitation and immune capture based kits (53), microfluidic devices (MFDs) (54-58), specific ligands/peptides for exosome binding and purification(59, 60), asymmetrical-flow field-flow fractionation (A4F) (61, 62), precipitation methods that have even been used in the clinic and ultrafiltration techniques (63) with or without subsequent size exclusion chromatography (SEC) or ionic exchange chromatography (64-67). Table 2 compares the characteristics of three selected purification techniques commonly used in the EV field today.

Potential associated issues	Purification method		
	Ultracentrifugation	**Density gradient centrifugation**	**Size exclusion chromatography**
High viscosity and hyperosmolarity	No	Yes/No*	No
Recovery	Operator dependent**	Operator dependent**	80% (66)
Loss of biological activity	Yes	Yes	No
Cause protein aggregation	Yes	No	No
Cause vesicle aggregation	Yes	No	No
Contaminating proteins and lipoproteins	Yes	Less	Less
Time for isolation	Around 180 min	6-72 hours	30-60 min

Table 2. Characteristics for the three main EV purification methods. *High viscosity and hyperosmolar media when sucrose is used, however Optiprep™ is isotonic in nature, but still has high viscosity. **Operator dependent and the reported recovery yield is typically low.

SEC has been shown to purify EVs devoid of 95% of the high density lipoprotein particles and removes 99% of all proteins, however one report showed co-purification of low density lipoprotein particles and EVs (48, 66). In the protein purification field, SEC is a well-established technique with many applications. The technique uses columns containing a porous gel-matrix with defined pore sizes and was developed in 1955 (68-70). Briefly, SEC works by trapping small molecules within the pores and allows for larger molecules to bypass, as they cannot enter the pores. Therefore molecules are separated based on their size (i.e. size exclusion), where the largest molecules in a sample elute first and smaller molecules

travel a longer distance through the pores of the column matrix, thus eluting later. To date, there are several different gel matrix materials and a range of pore sizes available for tailoring to the specific sample of interest. For EV purification relatively large pore sizes have been used (66), with one report showing the application of SEC columns for fractionation of differently-sized EV subpopulations (21). One major advantage with SEC is that the purity of EVs from both cell supernatants and biological fluids equals that of density gradient centrifugation. Furthermore, EV recovery rates are consistent up to 80% and EV functionality is maintained after purification (64, 71-74). Back in the 1980s, SEC was initially used for characterisation of EVs rather than purification (10), but nowadays there are even commercially available specific EV purification SEC columns which are used by several hundred labs world-wide (51).

Another purification method that separates vesicles based on size is A4F, which isolates particles via their diffusion properties and has been utilised to separate vesicles with 10 nm accuracy (61, 75), however it requires extensive optimisation and specialised equipment.

In a clinical setting, ultrafiltration followed by UC on a sucrose cushion as well as polyethylene glycol (PEG)-precipitation have been used (76-82), however the PEG-precipitation technique is rather poorly characterised. EVs purified with both methods were well tolerated by patients. Apart from PEG there are several other commercially available precipitation kits, such as ExoQuick™ from System Biosciences and Total Exosome Isolation™ from ThermoFisher Scientific, however the exact composition of the chemicals used to precipitate the EVs are not revealed by the manufacturers (49). Recently numerous other similar kits have been released. Importantly, these kits enrich EVs, however the process cannot be regarded as purification, since other protein aggregates and contaminants may also be precipitated (49).

MFDs have rendered considerable attention the last couple of years as the amount of starting material required for MFDs is commonly very low (<500 µl) and ideal for high-throughput screening of rare samples (83). MFDs can be organised into three categories based on their mode of action; 1) trapping EVs using immune-affinity, 2) sieving and 3) trapping exosomes on porous structures, whereas the most characterised type so far is the first category. In addition, one could develop customised MFDs containing functionalised surfaces with antibodies that can capture the EVs and directly analyse the readout by fluorescence. For instance, this technique was used to show that the fluorescence signal was stronger when pancreatic cancer patients serum were analysed compared with healthy controls (58). Another recent interesting development for MFDs utilises the combination of a functionalised surface and the use of surface plasmon resonance to detect binding to a functionalised surface through nano-holes (57). In this set-up, several different ligands with different affinity can be applied and the amount of target protein can be extrapolated due to the high sensitivity of the surface plasmon resonance technique. Hence, the surface protein composition of EVs can be determined. In conclusion, the MFDs are mostly developed for diagnostic purposes where they benefit from their small sample volumes and relatively low price.

So far there is still no consensus regarding the best purification technique, which further demonstrates the difficulties in assessing and comparing the results between different EV studies. It appears that the choice of purification method remains currently a compromise between purity, scalability and specific application, choice depending strongly on the sample type and posed research question.

1.5 EV COMPOSITION

Despite the challenges of EV characterisation, much is known about their overall composition. EVs have a lipid bilayer that resembles the plasma membrane, however certain lipids are enriched during the biogenesis, such as phosphatidylserine (PS), sphingomyelin and cholesterol (84). EVs contain proteins, RNAs (e.g. intact and fragmented messenger RNA (mRNA) (12-14), microRNA (miRNA) (85, 86), transfer-RNA- (87) and ribosomal RNA (rRNA) fragments (88) as well as long non-coding RNA (89), etc.), bioactive lipids and according to some recent reports DNA (11-14, 84, 90, 91). Due to EV biogenesis related sorting mechanisms, as more thoroughly described in the next section of this thesis below, a range of proteins are found to be enriched in EVs compared to the cell of origin, including tetraspanins, such as CD9, CD63, CD81 and CD82, heat shock proteins, programmed cell death 6 interacting protein (PDCD6IP) and tumour susceptibility gene 101 protein (TSG101) among others (15, 92) (See Figure 2 for a description of the composition of EVs). Importantly, these proteins are mostly considered to be enriched in the exosome fraction as compared to their parental cells.

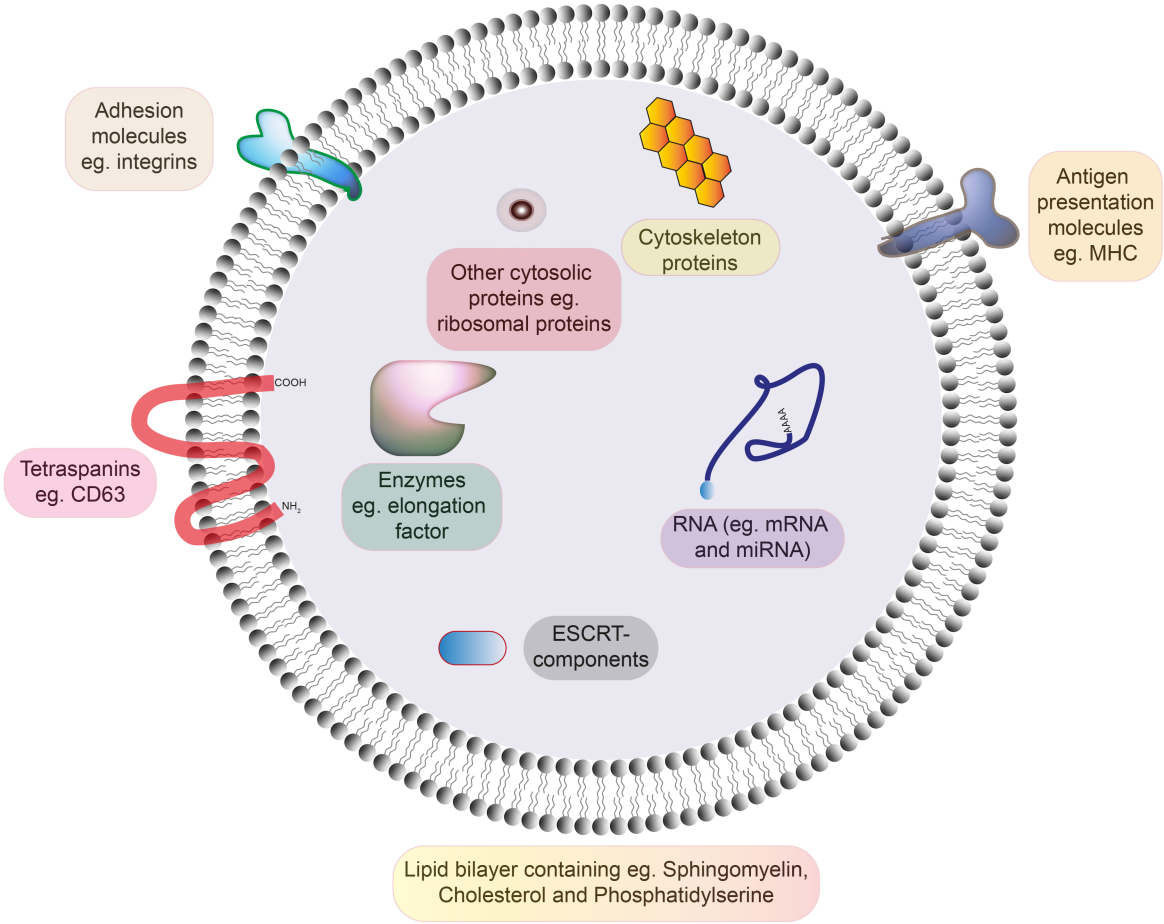

Figure 2. Overview of the content in Extracellular vesicles. Showing double lipid membrane with membrane proteins, soluble proteins and RNA loaded inside the vesicle. MHC: Major histocompatibility complex, ESCRT: Endosomal sorting complexes required for the transport

Proteins enriched in the MV fraction are less studied. One report suggests that β1 integrin is enriched in most MVs, whereas other highly enriched proteins in MVs appear to be cell-type specific (93), however some proteins materialise to have a rather uniform role in MV budding and possibly cargo sorting as well, as explained in the next section. Similarly to exosomes, also MV related proteins are devoid of proteins that are normally associated with intracellular compartments, such as endoplasmic reticulum and mitochondria, and serum proteins are normally not found in EVs (94).

Interestingly, regardless of the cellular compartment of origin, some cell specific proteins found in EVs have been proposed to be utilised as biomarkers for certain diseases, since EV composition changes in disease to a certain extent, mimicking changes in the diseased parent cell (95). Because membrane proteins in EVs have the same orientation as in the plasma membrane, protein-based biomarkers can potentially be analysed using already existing antibodies used in flow cytometry. Although there are a few highly enriched proteins, such as the tetraspanins, the majority of the proteins found in EVs are most probably stochastically sorted into the EVs and contribute to the high heterogeneity within an EV sample and the rich proteome of such sample.

The RNA content in EVs is another highly investigated topic, however to date there are no specific RNA markers found to be enriched in EVs derived from different cell lines similar to the tetrapanins for EVs. However the vast majority of the RNA found in EV samples is below 700 nucleotides (nt) (96), whereas mRNA length in cells can be up to 12 000 nt. There have furthermore been reports suggesting an enrichment of 3`untranslated regions of mRNA molecules (96). Commonly no intact rRNA is found in EVs (14), however some studies indicate that the majority of the EV RNA is rRNA fragments (88). Interestingly one study showed that the number of miRNAs molecules per vesicle is very low with less than one copy per vesicle. The most abundant miRNA molecule in a sample was less than one copy per 100 EVs (97). On the other hand, certain proteins at least when overexpressed can be as many as 40-50 molecules per vesicle according to FCS readings (personal communication G. Corso).

EV protein and RNA composition is highly complex and is specifically related to their biological activities. It seems that while certain EV cargoes reflect passively the changes of their parent cells, the presence of other cargoes depends strongly on the active sorting via interactions with the components important in their biogenesis, as explained next.

1.6 EV BIOGENESIS

The biogenesis differs for exosomes and MVs because of their different origin within the cell. As abovementioned, exosomes originate from the endocytic pathway and MVs are derived directly from plasma membrane budding (9, 93), however there have been reports about exosomes or exosome-like vesicles originating directly from the plasma membrane as well (51, 98). See Fig. 3 for a simplified scheme of the biogenesis of exosomes and MVs.

1.6.1 MVB formation and fate

The first phase in exosome biogenesis is the inward budding of the plasma membrane to generate an early endosome. From the early endosome stage the vesicle and the related material can take three distinct routes. The vesicle and its components can be recycled back to the plasma membrane, soluble intra-vesicle components secreted, and membrane recycled to the plasma membrane (99). The early endosome can also mature into a late endosome and in the process become an MVB by inward budding of the endosomal membrane that forms intraluminal vesicles (ILVs) (100). MVBs are 250-1000 nm in diameter and the ILVs around 30-100 nm, thus the same size as exosomes (101). While the process of inward budding to create ILVs starts in the early endosome (102), it is clearly enhanced in the maturing endosome. Later there are two fates of the developed MVBs, either the MVBs are degraded by fusing with a lysosome (103) or the MVBs fuse with the plasma membrane and the ILVs are released into the extracellular space as exosomes (11). The regulation of MVB fate is not

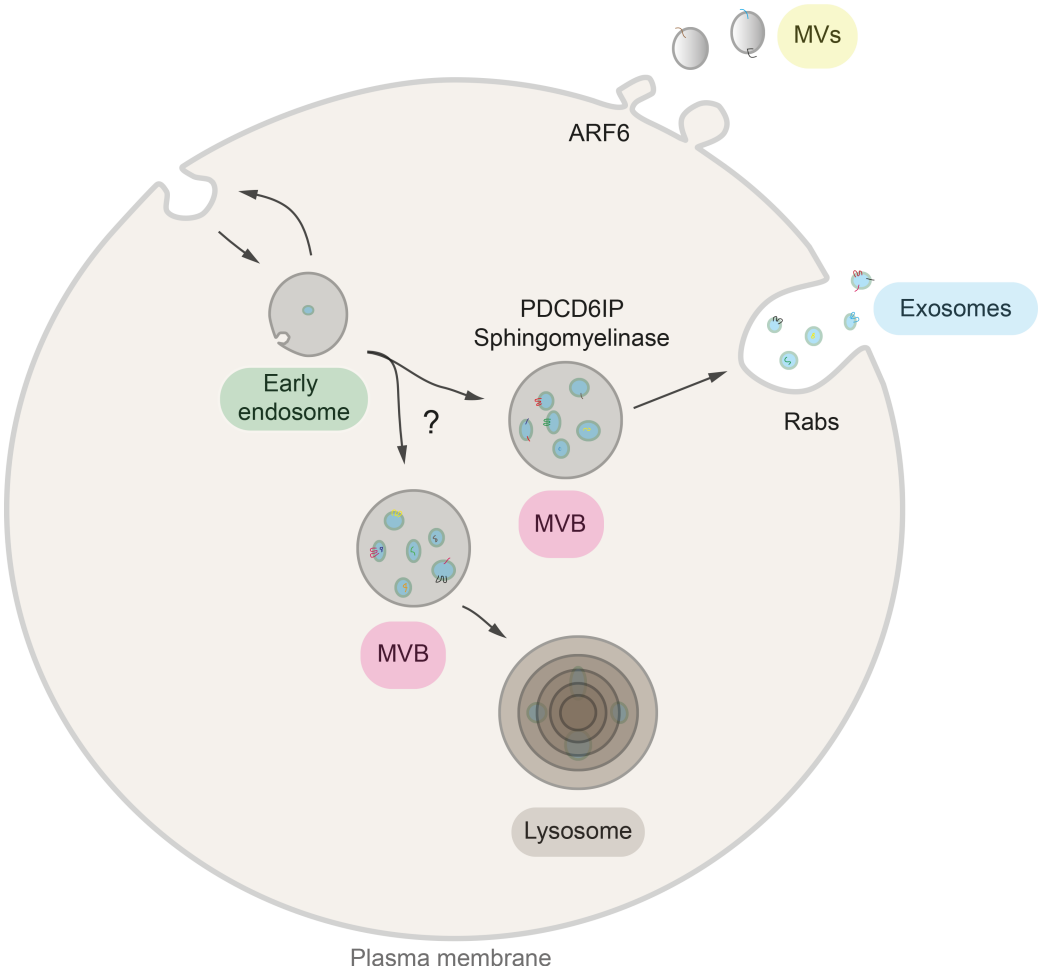

Figure 3. Schematic depiction over the biogenesis of Exosomes and Microvesicles. Showing MVB fate and key regulators of ILV loading and EV release. MVB: multivesicular body, PDCD6IP: Programmed cell death 6 interacting protein, ARF6: Adenosine diphosphate-ribosylation factor 6 and MV: microvesicle.

well understood, however it has been proposed that there are two subpopulations of MVBs in the same cell, one population destined for the lysosome and one for fusion with the plasma membrane. Two morphologically identical MVB populations with high or low cholesterol content have been identified, where the cholesterol rich MVB was more readily fusing with the plasma membrane as compared to the low cholesterol population that was rather destined for degradation (104). Furthermore, lysobisphosphatidic acid has been detected in lysosomal destined MVBs, although never in exosomes (105). While compelling evidence supports that there are two distinct populations of MVBs, it is however not clear what dictates the segregation of the two subtypes.

1.6.2 Protein sorting and loading into ILVs

The formation of ILVs and subsequent MVBs appears to be governed by several molecular mechanisms and the loading is thought to be highly regulated since exosomes have specific cargo proteins (15). The most investigated sorting mechanism to date is the endosomal sorting complexes required for the transport (ESCRT) pathway. The ESCRT pathway is divided into ESCRT complexes 0, I, II, and III. ESCRT-complexes 0, I and II are responsible for detecting and sequestering ubiquitinated membrane proteins on the limiting endosomal membrane. The role of ESCRT complex III is to take part in the membrane budding and scission of ILVs (106, 107). ESCRT-complexes also associate with auxiliary proteins such as PDCD6IP and vacuolar protein sorting-associated protein 4 that are involved in sorting of cargo into ILVs and disassembly of the ESCRT-III complex respectively.

The ESCRT-machinery is certainly important for the ILV loading of the MVBs directed towards degradation, however it is not clear how important the ESCRT-complexes are for the sorting of proteins to the ILVs in the MVBs directed for secretion. For example, there are several ESCRT subcomponents enriched in EVs, however only a small fraction of membrane proteins in EVs are ubiquitinated, unlike that of most cytosolic EV proteins (108, 109). Since ESCRT complexes detect and sequester ubiquitinated membrane proteins, most membrane proteins in EVs should, according to this hypothesis, be ubiqutinated if the ESCRT pathway was important for the loading of these proteins. Nevertheless, certain components of the ESCRT-complex have been shown to be particularly important for MVBs destined for secretion, such as PDCD6IP, which associates with the transferrin receptor in reticulocytes for the sorting of the receptor into exosomes and the subsequent, shedding of the receptor during erythrocyte development (110). Furthermore, PDCD6IP was discovered to take part in the sorting of syndecans into exosomes through association with syntenin (111). How cytosolic proteins are sorted into ILVs still remains relatively elusive, with one report suggesting an association with Heat shock cognate protein 70 (112).

Several other ESCRT-independent mechanisms have been found to be important for exosome biogenesis and protein sorting into EVs in recent years. For example, cells depleted of four subunits of the ESCRT-complex were still able to produce CD63-positive MVBs (113). In addition, sphingomyelinase, an enzyme responsible for the production of ceramide, has been shown to regulate exosome biogenesis and secretion in an ESCRT-independent manner (114). This correlates well with the high amount of ceramide and ceramide derivatives reported in exosomes.

Another important feature that could impact the sorting of proteins, for both EV types, is the curvature of the lipid membrane. The importance of the curvature for protein and lipid sorting has been studied both in artificial as well as eukaryotic membranes and it has been recognised that bacteria can sort proteins to certain micro-domains (115-117). Tetraspanins, have also been linked to induce membrane curvature and may contribute to protein sorting mechanisms by taking part in this biophysical pathway (118).

Certain tetraspanins, which are highly enriched in exosomes, have also been linked to mechanisms directly controlling the sorting of proteins into ILVs (119). Possibly related to some functions of tetraspanins, there have also been reports that physical clustering of different proteins is important for exosome secretion. For example, the secretion of the transferrin receptor, major histocompatibility complex II (MHC-II) molecule and CD43 via exosomes increased after antibody crosslinking in reticulocytes, lymphocytes and Jurkat-cells, respectively (98, 120, 121). Hence, there appears to be both an ESCRT-dependent and an ESCRT-independent pathway for the biogenesis and protein sorting into ILVs.

1.6.3 Control of MVB fusion and exosome release

Several different cellular components are required for the transport of MVBs to the cell membrane and the subsequent fusion with the plasma membrane and release of the ILVs. The transport of the MVB requires active involvement of the cytoskeleton and its active transport mechanisms. The fusion of MVBs with the plasma membrane most likely involves members of the SNARE-family, although the exact members of these SNARE components have yet to be identified. Furthermore, several studies suggest small Rab-GTPases as key regulators in the secretion of exosomes as models with knockdown of Rab11, 27a and 27b or their effector proteins result in significantly lower amounts of exosomes released (122, 123).

1.6.4 MV biogenesis

If the biogenesis and sorting mechanisms appear unclear for exosomes, the picture is even more ambiguous for MVs. Similarly to exosomes, small GTPases and other cytosolic proteins, such as Adenosine diphosphate-ribosylation factor 6 (ARF6) and TSG101, may assist the recruitment of other proteins to the plasma membrane and affect the regulation of MV budding (44, 124). The biogenesis of MVs has furthermore been shown to be a result of phospholipid redistribution in the plasma membrane and cytoskeleton contractions. The phospholipid content in the plasma membrane is not homogenous and forms microdomains together with proteins. An increase of phosphatidylserine in the outer-membrane leaflet induces MV formation and contractions in the cytoskeleton completes the process (125). To summarize, the biogenesis and protein loading of MVs is not fully understood and requires more research.

1.6.5 RNA sorting and loading

While more information is available regarding protein loading, the sorting of RNA-species into EVs remains unclear. In certain cell lines, "zip-code" sequences have been identified in EVs (126). However to my knowledge, there is still no ubiquitous RNA sequence that can be found in EVs from across all cell types. For small RNA species, some have suggested that the presence of RNA-binding proteins may aid their enrichment in EVs. For example, Melo *et al* showed that breast cancer EVs were loaded with miRNAs associated with the RNA-induced silencing complex (RISC) and these EVs had the capacity to process precursor miRNAs into mature miRNAs independent of their cellular origin (127). This process may be dependent on activation status of a particular RISC component protein, as phosphorylated argonaute 2 inhibited miRNA secretion via EVs (128). Sumoylated hnRNPA2B1, another RNA-binding protein, can control the loading of specific miRNAs containing the sequence 'GGAG' into EVs (129). Furthermore Y-box protein 1 has been implicated in the loading of miRNAs into EVs, in cells as well as in a cell free reaction (130). From the data to date, the loading of both proteins and RNAs into EVs appears to be tightly regulated by the parental cell and that multiple mechanisms can be active simultaneously. However, the exact mechanisms for the sorting of both RNAs and proteins and whether these pathways are consistent across cell types remain to be elucidated.

In summary, the biogenesis of EVs is a complex process that requires several different cellular components. The sorting of proteins into EVs can be dependent on the ESCRT machinery as well as through ESCRT-independent mechanisms. The biogenesis is not fully investigated and especially MV biogenesis needs further clarification to fully unravel the mechanisms that govern EV generation. EV biogenesis is further important for the understanding of EV interaction with the surroundings that will be discussed in the next chapter.

1.7 EV INTERACTIONS *IN VITRO* AND *IN VIVO*

1.7.1 Effects on recipient cells and uptake mechanisms

EVs can be seen as advanced signalosomes that can affect recipient cells in a number of ways. Surface proteins on EVs, such as receptors and ligands can in their own right prompt downstream signalling cascades in cells residing within the vicinity of these EVs. Alternatively, after EV internalisation, intra-vesicle proteins can interact with intracellular receptors. Another mechanism how the EVs can influence recipient cells is by transferring functional receptors onto recipient cells, such as C-C chemokine receptor 5 (CCR5) and epidermal growth factor receptor (EGFR) vIII and thereby changing the signalling capability

of the cell (131, 132). Furthermore, the RNA content of EVs plays an important role after uptake to induce changes in the cellular gene expression profile. The first articles showing RNA transfer between cells demonstrated that mRNA can be transferred to and translated by recipient cells into functional proteins (12-14). Similarly, there are many later studies showing that miRNAs from EVs can induce wide alterations in the epigenetic and protein expression profile of cells (85, 133). However, the degree of influence the mRNA and miRNA content has on the behaviour and proteome of recipient cells is variable and unclear.

Since many of the actions of EVs on recipient cells described above require EV internalisation, the uptake mechanisms of EVs have been a rapidly expanding field. Generally, the uptake of EVs appears to be mostly by endocytosis, with some reports on pinocytosis by certain cell types (134, 135). A recently published study revealed with elegant microscopy techniques that EVs are taken up as single vesicles and that before internalisation they 'surf' on filopodia. The uptake appeared to be highly effective and a fast phenomenon that resembled the way viruses are taken up by recipient cells (136). The uptake can furthermore be mediated by Heparan sulfate proteoglycans (HSPG), which has been reported to be important for the uptake of cancer cell derived EVs. Cells with low amounts of proteoglycans were shown to take up 2.5 fold less EVs compared to wild type cells and the uptake was reduced by 50% when HSPGs were enzymatically depleted (137). Furthermore, several groups have demonstrated that heparin blocks EV uptake in several cell lines by 50-80% (59, 137, 138).

Another school of thought governs the possibility of membrane fusion at conditions where the cell and EV membrane have the same fluidity. In this instance, the microenvironment would play an important role as both membranes appear to have similar fluidity at slightly acidic conditions around pH 5 (139), which would enhance the probability of fusion (140). This mechanism is thought to be viable for example in tumours where the pH is generally lower. It should also be noted that the pH in MVBs is around 5 and that ILVs have been shown to back fuse with the MVB limiting membrane (141), thus supporting membrane fusion of EVs.

Currently gathered information suggests that similarly to the diverse range of EV-associated bioactive molecules and effects, also the interaction mechanisms with their target cells can be very varied. This may again reflect the highly heterogeneous nature of secreted vesicles whose functions can depend on specific conditions.

1.7.2 Influence of EV surface proteins on biodistribution

Most studies regarding biomolecular effect mechanisms of EVs have been highly informative but have been performed *in vitro*. However, to understand EV effects *in vivo* this knowledge is insufficient because it does not fully reveal what defines their site of action in an organism. In order to understand the latter, it is important to study how EV surface proteins affect their biodistribution profile. This is related both to the EV clearance from blood and the subsequent distribution in the extracellular matrix, which could be essential to their subsequent biological functions. For example, integrins on tumour-derived EVs have been shown to influence their biodistribution in mice. Depending on their integrin repertoire, these EVs home to different organs and induce a pre-metastatic niche, thus enhancing the spread of metastasis (142). Alternatively, other adhesion molecules have been shown to be important for the biodistribution. For example, α2,3-linked sialic acids exposed on certain B-cell derived EV surfaces can bind to CD169 and regulate the uptake into the spleen and lymph nodes (LN) (143). This was further verified when CD169 knock-out mice showed a

dysregulated EV trafficking to the LN cortex (143). Another important protein for uptake and biodistribution of EVs is Milk fat globule-EGF factor 8 (MFGE8), which binds PS on apoptotic cells and EVs. Upon binding to PS, the protein undergoes a conformational change (144, 145), which can facilitate the binding of EVs to macrophages expressing αvβ3 and αvβ5 integrins and the subsequent phagocytosis of the EVs or apoptotic cells.

Hence, a range of different surface molecules influence the biodistribution of EVs, however the uptake *in vivo* of exogenously administrated EVs appears to be sequestered by immune cells in the mononuclear phagocytic system (MPS), as expanded further in the next chapter.

1.7.3 MPS contribute to EV uptake *in vivo*

In a bid to further understand the biodistribution of EVs, EVs have been labelled and re-administered in various mouse models. Interestingly, DiR (1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide) labelled EVs injected by tail vein injection in NOD.CB17-Prkdcscid/J mice, which have a compromised innate immune system as well as complement system, had slower EV uptake in the liver and spleen as compared to nude mice, which have a compromised adaptive immune system, and Balb/c mice. Therefore, this dataset indicated that the complement system as well as the innate immune system may impact on the uptake of EVs in the MPS (146). These findings also suggest that EVs are rather similar to other lipid nanoparticles which also display complement receptor mediated uptake (147).

Alternatively, PKH26 labelled B16-melanoma derived EVs were taken up by macrophages in the liver and spleen however, not in lung, where the EVs appeared to be predominantly taken up by endothelial cells (148). Another study found that the clearance from blood of Gaussia luciferase labelled EVs was slower in macrophage-depleted mice (148). Moreover, one study showed that the uptake of EVs in macrophages was inhibited by dextran sulphate (149), a scavenger receptor A inhibitor, which is in accordance with other nano-sized particles that are also taken up via scavenger receptors (150). Furthermore, dextran sulphate reduced the uptake in liver by 50% when it was co-administrated with the EVs, which subsequently enhanced the uptake of EVs in an implanted subcutaneous tumour in the same mice (149).

Hence, compelling evidence support macrophages in the MPS to be important for the uptake of EVs intravenously (IV) administered, which may be mediated by MFGE8, scavenger receptors, HSPGs and/or complement factors.

1.7.4 EV clearance from blood

The clearance of EVs from the blood circulation has been shown to be rapid after exogenous administration; this can range from as little as 10 to 60 minutes (151-154). Additionally, less than 5% of radiolabelled tumour derived EVs injected in nude mice were found in the blood 3 hours post injection (146). On the other hand, platelet derived EVs were found to have a longer half-life in blood of 5.5 hours (155). Besides the origin of EVs, the biophysical characteristic of EVs, for example PEGylation, can also increase their half-life in blood. Control EVs were cleared from the circulation within 10 minutes, whereas PEGylated EVs were still detected 60 minutes after injection (156). Thus, there is a large disparity in the reported clearance of EVs, dependent on the type of EVs and model systems used. It is proposed that the clearance of EVs from blood is unlikely due to lysis (157), but rather dependent on uptake into target organs and most particularly uptake by the MPS as described in chapter 1.7.3. To add on, this disparity in the uptake of the nanometre sized particles for the different organs may also be linked to the microstructure of the capillaries. Several studies have shown that nanoparticles under 100 nm are less prone to be affected by opsonisation and

can penetrate the fenestrated endothelium in the liver and can also extravasate in the spleen due to the discontinuous endothelium (158-160), thus increasing the uptake of nanoparticles in spleen and liver.

In addition to the nature of EVs and model systems, we have found that the biodistribution of EVs can also be dependent on the purification technique, since the purification technique can influence the integrity and the purity of the vesicles. This data will be further described in detail in chapter 4.1. Contrary to our study, another group found that the use of 3 different purification methods: UC, Optiprep™-cushion or an Optiprep™-gradient, did not appear to influence the clearance of EVs (161). However, the authors found that EVs were recovered to a greater extent when the purified EVs were later 0.2 μm filtered after purification of an Optiprep™-gradient compared to UC purification (82% versus 50%), again indicating that UC causes aggregation of the EVs.

To sum up, many studies have concluded that the half-life of EVs in blood is relatively short, however a few studies have demonstrated that specific EVs have longer half-life of up to several hours and PEGylation overall prolongs the half-life of EVs. How technical differences in purification and/or innate capabilities of the particular EVs investigated may affect clearance has yet to be determined.

1.8 BIODISTRIBUTION EVALUATION STRATEGIES

1.8.1 Chemiluminescense strategies for EV biodistribution

The biodistribution of EVs has been evaluated using several different labelling strategies, including radioactive probes, fluorescent dyes and chimeric biotionylated/strepavidin and chemiluminescence proteins (146, 152, 154, 162). For example in the chimeric Gaussia-luciferase probing method, an EV targeting domain is fused with Gaussia luciferase, allowing for the tag to be enriched in EVs. One such EV targeting domain that has been used is the C1C2 domain of MFGE8 (148, 152) and another is the membrane part of the platelet-derived growth factor (PDGF)-receptor (154, 163). When the PDGF receptor-Gaussia construct was used to label HEK293T EVs, the EVs appeared to accumulate mostly in the spleen followed by liver, lungs and kidneys 30 minutes after administration. On the other hand, the brain, heart and muscle all exhibited a relatively low signal throughout all measured time points (154). Interestingly, up to 50% of the signal in the spleen was retained after 360 minutes, as compared to the initial 30-minute time point, whereas signals in the lungs and liver fell to under 15% and 5% respectively.

Likewise, B16-BL6 derived EVs labelled with the MFGE8 construct presented a similar tissue profile, although the most intense signal originated from the liver followed by lungs and spleen from the 10 to 60 minute time points, although at the longest time point (4 hours) signal was only detected in the lungs during whole animal scans (152). After the organs were harvested at 4 hours, luciferase activity was merely detected in lungs and spleen. Importantly, in both studies, the authors verified that these signals were resultant of true EVs by using sucrose gradient or SEC fractionation to characterise EVs. While the biodistribution is noted to be similar between studies utilising Gaussia luciferase to label EVs, there were still some important differences between the studies. The discrepancy in distribution between HEK293T EVs, that primarily distribute to spleen and liver, and B16-BL6 EVs that more readily accumulate in lungs is difficult to interpret. There are a number of reasons that can account for these differences; first, these EVs are derived from two different cell lines from two different species with varied biological characteristics and second, different EV loading proteins were used, which may only label certain subpopulations of EVs. Furthermore, the

mice used in these studies were different; HEK293T EVs were injected in immune compromised athymic nude mice, whereas the B16-BL6 EVs were injected in immune competent BALB/c mice, and different doses were used (100 μg of HEK293T EV- compared with 5 μg of B16-BL6 EV-protein).

1.8.2 Fluorescent probes utilised for EV biodistribution

Apart from chemiluminescence, many different fluorescent dyes and fluorescent probes have been utilised to track EVs in animals. To date, for global biodistribution studies *in vivo* DiR, a commercially available lipophilic dye with good *in vivo* features, such as emission at near infra-red wavelengths, has been the most widely used. For example, one study utilised DiR to examine the biodistribution of unmodified tumour derived EVs as compared to PC:Chol liposomes and liposomes mimicking the lipid composition of EVs (146). In all cases, the EVs/liposomes were readily taken up by liver and spleen, with no or weak signal originating from orthotropic tumours implanted in the mice. However when the formulations were injected intra-tumouraly, the EVs remained associated with the tumour tissue longer than that of PC:Chol liposomes. Furthermore, it was shown that the biodistribution in tumour bearing mice and non-tumour bearing mice appeared similar, thus, the addition of a tumour did not have any impact on the overall biodistribution of exogenously administered EVs or liposomes. Another interesting finding of this study was that mice receiving the highest dose (400 μg EV protein) had signs of asphyxia and recovered slowly. When the authors tried to elucidate the cause for the shortness of breath, these mice did not recover and died 3 minutes after the injection. Subsequently, the necropsy found a high accumulation of EVs in the lungs, suggesting that the EVs were trapped in the lungs, which caused the symptoms.

DiR has also been used to study the biodistribution of tumour targeted doxorubicin (Dox) loaded immature DC EVs (164). These EVs were targeted to tumours by endogenously expressing a fusion construct of iRGD with Lamp2b, a reported EV marker, in the parental EV producing cells. Strong DiR signal was found in the tumour tissue after 30 minutes and peaked around 2 hours after IV injection of iRGD EVs. In contrast, no signal was detected in the tumours when non-targeted EVs were injected. When the organs were harvested and analysed two hours post injection, the strongest signal was observed in tumour, liver and spleen for the targeted EVs whereas non-targeting EVs localised to liver and spleen, but not to the tumour. Importantly, administration of these Dox loaded targeted EVs led to the reduction in the growth rate of MDA-MB-231 tumours *in vivo* compared to free Dox and EV controls, aptly showing how biodistribution results corresponded with biological readouts. In another study, EGFR-targeted EVs for tumour treatment were labelled with DiR and injected IV (165). Although the global biodistribution did not appear to change, with the EVs predominantly taken up by the liver, EGFR-targeted EVs were found to be taken up in tumour tissue 3 times more than control EVs. The EGFR targeted EVs loaded with Let-7a miRNA were also shown to supress tumour growth in an orthotropic tumour model.

Other commercial fluorescent dyes have been used to investigate more specific EV distribution enquiries. For example, PKH26/67 has been another commonly used dye to track tumour EV uptake in organs in several studies and/or track therapeutically active EVs to their site of action (142, 165-170). One study showed the uptake of EVs after intranasal delivery where they separately studied the delivery of MVs and exosomes. Interestingly, the exosomes were found in the brain and intestine, whereas the MVs distributed mostly to the lung. On hindsight, free dye also distributed mostly to the lungs (170).

In studies using fluorescent dyes, there has yet to be any experiment clearly showing that it is only the EVs that are labelled or that all excess dye is completely washed away; which makes the results difficult to interpret. Furthermore, when using these unspecific lipid dyes, it is unclear which specific group of EVs that are labelled and to what extent the proportion of the vesicle pool is actually labelled.

1.8.3 Other biodistribution strategies

Another biodistribution strategy was employed in an attempt to overcome the latter issue: to determine the tissue distribution of EVs Bala *et al*. used a certain miRNA for biodistribution studies. Murine B-cell derived EVs were loaded with miRNA-155 mimics by electroporation and injected IV in miRNA-155 knockout mice (171). The distribution of miRNA-155 was subsequently analysed by qPCR in perfused organs and found to be highest in liver, adipose and lung tissue and lowest in muscle and kidney. The uptake in spleen was not analysed in this study. The miRNA was also found to be cleared rapidly from the circulation similar to other exogenously injected EVs described earlier.

To sum up, all these described labelling methods most probably show the distribution of EVs. Unfortunately, these methods do not indicate whether the EVs are functional in the targeted organs they home to. Recently, Cre mRNA has been shown to be loaded into EVs and this has been used to demonstrate functional transfer of cancer EVs *in vivo* (172), however, the technique has not yet been utilised to examine the biodistribution of exogenously injected EVs. This is difficult because even in *in vitro* co-culturing systems, this recombinase system is highly inefficient with successful recombination in around 1-5% of the recipient cells (172). Nevertheless, the potential of this system was demonstrated in a study in which mice expressing Cre recombinase only in the hematopoietic lineages had cells showing recombination in the brain. The number of recombined cells in the brain increased after systemic inflammation. The authors furthermore found that the EVs from the hematopoietic cells contained Cre mRNA that induced recombination when injected in the brain (173).

To conclude, several different strategies to evaluate the biodistribution of EVs have been used during the last decade where many studies rely on chemoluminesense and near-infrared fluorescent probes. Although these different studies have used different EV sources and labelling techniques, the general consensus is that uptake of exogenously IV injected EVs follows a similar pattern; with the majority of the EVs rapidly taken up by liver, lung and spleen.

1.9 EVS IN HEALTH AND DISEASE

1.9.1 The good EVs (EVs in physiology)

As described earlier, EVs contain several key biological components, such as receptors/ligands and other membrane and cytosolic proteins, a range of biologically active RNAs and bioactive lipids (15). Hence, EVs can influence recipient cells in a number of ways depending on their bioactive cargoes and having distinct biological consequences during both physiological as well as pathological conditions. This seems to be a general organism-wide mechanism, as EVs have been detected in several body fluids including blood, urine, cerebrospinal fluid, saliva, bile, breast milk, amniotic fluid and seminal fluid (92).

Physiological events that EVs have been implicated in are remarkably diverse, such as blood coagulation, both the innate and adaptive immune response, neuronal communication, reproduction, embryonic development, tissue repair, bone calcification, liver homeostasis and

reticulocyte maturation. It should be noted however that the evidence for the physiological importance of EVs in some organ systems is stronger than in others. Examples of well-known cases where EVs take part in physiological events are discussed below.

Perhaps the best-described physiological role of EVs are related to the functions of the immune system. EVs have been shown to be important players for both immune activation and in tolerogenic processes. A key publication in the EV field from Raposo *et al.* showed that B-cells released EVs with peptide loaded MHC-II molecules, which could in turn activate T-cells (11). Furthermore, DC derived EVs have been shown to elicit immune responses and eradicate tumours in mouse models after systemic injections (174). However, immature DC EVs have shown tolerogenic effects in both an arthritis model and in a skin graft survival model (175, 176). Non-immune cell derived EVs seem to affect the immune system as well. For example tumour cells secrete EVs that supress inflammatory pathways via a number of different mechanisms including enhancement of regulatory T-cell function, suppression of natural killer cells as well as $CD8^+$ T-cells (177-179). Additionally, mesenchymal stromal cell (MSC) EVs can polarize macrophages to an M2 phenotype further extending the immunosuppressive repertoire of EVs (180).

Another organ system where EVs are important is the nervous system where EVs take part in processes such as neurite outgrowth, neuronal survival and synaptic plasticity (181-183). EVs released from oligodendrocytes as well as Schwann cells have been shown to be protective for nerve cells in the peripheral- as well as the central nervous system and partly regulated by electrical activity and neurotransmitter release (184). Stem cell derived EVs have also been highlighted as important for the stem cell niche formation as well as in tissue regeneration (185, 186). The regenerative capabilities of EVs have been utilised for therapeutic purposes discussed in more detail in chapter 1.10.

One area under intense investigation is the role of EVs in the coagulation process. EVs have mostly been described as pro-coagulant (1). Mostly larger vesicles have been attributed with pro-coagulant characteristics, however studies have shown that smaller EVs can also carry tissue factor and hence be pro-coagulant as well (187, 188). Additionally, Scotts syndrome which is a bleeding disorder with decreased platelet function has been shown to have a reduced phospholipid scramblase activity, which leads to decreased release of pro-coagulant vesicles and low pro-thrombinase activity (189).

EVs take part in numerous physiological events from embryonic development to neuronal communication and are today seen as important carriers of intercellular messages in order to take part in orchestration of these events.

1.9.2 The bad EVs (EVs in pathology)

Since EVs have such a broad range of effects and are important for many organ systems, it is no surprise that they have been linked to several pathological processes, such as cancer, spread of infections and prion disease as well as several neurodegenerative diseases when their source cells are defective in one way or another. For example, several publications in the last couple of years have highlighted the role of EVs in cancer development and especially in the metastasis of different cancers.

Research from Professor David Lyden's group have helped to pinpoint key steps in the metastasis of both melanoma and pancreatic cancer where EVs are key players in the initial steps of forming the pre-metastatic niche (168, 169). In melanoma, EVs released by

melanoma cells polarize bone marrow derived cells to a more pro-metastatic phenotype as well as promoting them to migrate to future metastatic organs, such as the lungs, through the horizontal transfer of the MET protein. Furthermore, EVs do directly influence the organs prone for metastasis of melanomas, for example by enhancing the leakiness of the lung vasculature, a phenomena seen early in metastatic melanoma (169). Additionally, pancreas adenocarcinoma derived EVs influence Kupffer cells to secrete transforming growth factor-β, which induces hepatic stellate cells to produce fibronectin and the resulting fibrotic microenvironment increases the recruitment of bone-derived macrophages, which in turn help to form a pre-metastatic niche for the pancreas adenocarcinoma cells (168). Moreover, in mouse models of both melanoma and pancreas adenocarcinoma, the infusion of cancer cell-derived EVs increases the metastatic burden significantly even in cancers that have normally low rates of metastasis (168, 169).

In addition to the role in metastasis formation, tumour EVs can suppress normal defence mechanisms of an organism. Melanoma EVs are commonly drained to LNs where they can interact with different immune cells, however there is a subscapular LN layer of macrophages that guard the LNs and hinders the melanoma EVs to reach deeper layers of the LNs (163). During tumour progression this macrophage layer is disrupted and the EVs can penetrate the LNs and interact with other immune cells and prompt a pro-tumour immune response by inducing the production of tumour-promoting antibodies. EVs have also been implicated to promote tumour progression in several other ways including, immune escape by altering T-cells, secretion of metalloproteases which leads to matrix remodelling and directly stimulating tumour growth (13, 190, 191). Hence, the interaction between tumour EVs and the surrounding cells and tissue is highly complex and macrophages as well as other immune cells act in both a tumour suppressive and tumour promoting way after engagement with tumour EVs.

In neurodegenerative diseases EVs have been implicated to facilitate the spread of toxic proteins. Both α-synuclein and amyloid-β-peptide have been found in EVs and enhance the spread of these proteins (192, 193). However, possibly depending on the parent cell or specific EV components, in some cases EVs can aid in toxic protein clearance as well (194). Certain viruses also take advantage of EVs for their dissemination, such as human immunodeficiency virus that utilises the fact that EVs can horizontally transfer the CCR5-receptor to non-immune cells, which the virus utilises for entry (132).

EV research regarding disease development is very intense and will hopefully in the coming years lead to new therapies against cancer and other diseases when our understanding of the EVs' contribution to the disease burden increases. Some success has already been made in this field, as summarized in the next section.

1.10 EVS AS THERAPEUTIC MOIETIES

Given EVs' functions both in physiological and pathological processes, utilising native or modified EVs for therapy has been recognised as a great opportunity. Furthermore, modulation of pathogenic EV-related processes by drugs can have significant value for therapy as well. The on-going research into therapeutic EVs is focusing on both utilising the innate capabilities of EVs as well as to engineer them for specific purposes.

A great example of applicability of native EVs came from the field of cell therapy. MSCs have been used to treat several diseases in humans, since they have innate immunomodulatory capabilities. However no or very little engraftment of the cells was

detected after cell therapy, hence the cells did not replace dying or diseased tissues (195). Therefore, researchers started to investigate if paracrine factors could explain the therapeutic effect seen after MSC cell infusions. The first articles that reported that MSC derived EVs were responsible for the therapeutic effect seen by MSCs emerged in 2009 when the therapeutic effect of MSC EVs in animal models of acute kidney injury and acute myocardial infarction was shown (196, 197). Since then, the intrinsic immune-modulatory effects of MSC EVs have been utilised to treat several other disease animal models including liver failure, acute lung injury and ischemic limb disease (198-200). Perhaps the most significant example showing the therapeutic effect of EVs is the successful treatment of a human patient suffering from Graft versus Host disease using MSC EVs (80).

Another interesting EV source is DCs. DC EVs primed with cancer antigens are investigated as a cancer vaccination strategy and clinical trials have been performed. Early results indicate that the treatment is safe, however the therapeutic effect was modest, although all trials had a few patients with stabilisation of the disease after therapy (201). The modest effect can be due to immune-regulatory mechanisms such as programed death ligand 1 expression and/or insufficient stimulation of the T-cells by the EVs. Interestingly, another clinical trial in China used EVs derived from ascites fluid from colorectal cancer patients and injected the EVs together with granulocyte-macrophage colony-stimulating factor to stimulate a DC response against the tumour *in vivo* (79). The study found that the treatment was safe and that the treatment could elicit tumour specific cytotoxic T-cells.

In line with the benefits of using EVs innate capabilities for therapy, another very intriguing approach is to engineer EVs into a potent drug carrier for therapy. This includes strategies such as loading them with specific RNAs, proteins and/or to add targeting moieties to their surface for specific tissue targeting. Early work showed that cells transfected with a miRNA-mimic, secreted EVs containing this miRNA-mimic, which could be subsequently transferred to recipient cells (202). Later, it was shown that siRNA could be loaded into EVs by electroporation (166). The authors also showed that the siRNA loaded EVs could be targeted to brain with a brain targeting peptide by transfecting the producing cells with a chimeric construct consisting of a brain targeting peptide fused to Lamp2b. After IV infusion of the siRNA loaded EVs, the delivered siRNA down regulated genes in the mouse brain in a sequence-specific manner. Electroporation as a mean to load EVs has been employed by several other labs after the first publication (167, 203-205). However, one caveat is that this loading strategy is very inefficient because electroporation leads to precipitation of siRNA into crystallised structures that prevent their entry into EVs (206). Nonetheless, there are several new publications showing that EVs can still be engineered and exploited for therapeutic endeavours. This field of research is still young and many obstacles still remain before the technology enters the clinical arena, such as, large scale production and purification of modified EVs and specific potency assays that can be employed for quality assessments of the EV product.

2 AIMS

The aims of this thesis are related to several crucial issues in the EV research field. Firstly, purification methods for isolating pure and intact EVs needed significant improvement. Secondly, methods to assess EV biodistribution in an unbiased fashion needed to be established. This led to the postulation of individual specific aims of each individual publication of this thesis, as stated below.

2.1 PAPER I

- To compare ultrafiltration with subsequent size exclusion liquid chromatography with ultracentrifugation for the purification of EVs, especially regarding the yield and purity of the EVs.
- To investigate the intactness of the vesicles after purification, since UC purification takes place under extreme *g*-forces.

2.2 PAPER II

- To find a method to explore the biodistribution of EVs without the need for genetically engineering the producing cells or selectively label specific subgroups of EVs.
- Compare the biodistribution of EVs from different cellular sources.
- Investigate whether the route of administration or dose influences tissue distribution.
- Utilise the method to study how targeting moieties attached on EVs can change the biodistribution in mice.

2.3 PAPER III

- To combine size exclusion- with bind elute-liquid chromatography to further stream line the workflow during EV purification.
- To develope a purification method that would be easy to scale up and at the same time keep the vesicles intact and produce a pure sample.

3 METHODOLOGY

3.1 METHODOLOGICAL CONSIDERATIONS

A full method description is available in the attached articles and manuscript. Here, a brief outline of the most important methods will be presented.

3.2 CELL LINES

The cell lines; NSC-34, a fusion of motor neuron enriched embryonic mouse spinal cord with mouse neuroblastoma, Neuro2a (N2a), a mouse neuroblastoma, B16F10, a mouse melanoma, HEK293T, human embryonic kidney, C2C12, a mouse muscle, and OLN-93, a rat oligodendrocyte, were used. Cells were cultured at 37°C with 5% CO_2 in complete media comprised of Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) except for C2C12 which had 20% FBS supplemented, and penicillin/streptomycin (P/S, 5000 µg/ml). The conditions used during induced pluripotent stem cell and primary cell cultivation is described in the attached articles and collaborators cultivated the cells. For EV isolation, the medium was changed 24 hours post seeding to either OptiMEM or pre-spun media. Conditioned medium was collected after 48 hours of incubation.

3.3 EV PURIFICATION

EV purification in the three studies has either been by UC, ultrafiltration UF w/o SEC or bind elute combined with SEC (BE-SEC). The most common media to purify vesicles from in this thesis were cell supernatants. Both for UC and filtration purification methods the conditioned media has been processed before the final purification with a series of centrifugations and filtration steps. Firstly the media has been centrifuged at 300*g* and thereafter 1500-2000*g* to get rid of cell debris. Thereafter a 0.2 µm filtration step has been undertaken to separate the larger vesicles from vesicles under 200 nm. Lastly the media has been spun at 110 000*g* for 70 minutes and the pellet re-suspended in PBS and spun again for 70 minutes in PBS at 110 000*g* or the media have been ultrafiltrated using commercially available 100 kDa spin filters or with tangential flow filtration devices (100 and 300 kDa filters used) and the retentate has been further purified by liquid chromatography (LC) on Sephacryl columns or BE-SEC columns (CaptoCore 700), all from GE healthcare (Sweden).

3.4 WESTERN BLOT

WB is used to detected proteins of interest in a sample by applying primary and secondary antibodies for the visualisation of the specific protein. In short, the sample was mixed with a sample buffer to solubilise and denature the proteins. In the sample buffer sodium dodecyl sulfate dissolves hydrophobic regions of the protein as well as breaks non-covalent ionic bonds. The β-mercaptoethanol or ditiothreitol is in the sample buffer to break up disulfide bonds. Lastly, the sample was heated to completely denature the protein. The proteins were then separated by gel electrophoresis and transferred to a nitrocellulose membrane, which was blocked by adding blocking buffer. The membrane was then stained with primary and secondary antibody and analysed by LI-COR Odyssey CLx infrared imaging system.

3.5 NANOPARTICLE TRACKING ANALYSIS

NTA was used to measure the particle concentration and size of the EV samples on the Nanosight NS500 (Malvern, UK). The NTA measures the particle size based on Brownian motion, which is the random movement of particles suspended in solution. The particle size can be calculated using the Stokes–Einstein equation. The instrument uses a laser to make

particles between 30-1000 nm scatter light that a CCD camera can detect and the computer software can then follow the Brownian motion of individual particles and hence calculate the size and concentration. The samples analysed were first diluted to achieve a particle count between 2 x 10^8 to 2 x 10^9 particles per mL. The script control function of the software was then used to run the samples and the batch process function used to analyse all samples. The setting was maintained throughout one experiment.

3.6 TRANSMISSION ELECTRON MICROSCOPY

TEM was performed in each study to verify that we indeed had vesicles in our samples that resembled EVs. A 2% uranyl acetate solution was used to stain the grids to visualise the vesicles.

3.7 LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS)

LC-MS/MS was done in collaboration with a group at SciLife lab at Karolinska Institutet in Solna. LC-MS/MS is done to elucidate the protein profile of a sample. The sample is denatured and cleaved to peptides by trypsin. The peptides were then analysed by LC-MS/MS and GO-term enrichment was done by Panther analysis (207).

3.8 FLUORESCENCE CORRELATION SPECTROSCOPY

FCS was done by collaborators at Novartis in Basel, Switzerland. FCS can measure single fluorescent molecules and how they move in solution. Hence the Brownian movement can be analysed and the size of the molecule be deducted. Furthermore, the fluorescence intensity of each particle can be measured. Here we utilised FCS to measure vesicles in solution to determine the concentration, size and fluorescence intensity per particle. From the fluorescence intensity, green fluorescent protein (GFP) molecules per particle can be calculated. The particles were measured both with and without NP40s treatment. NP40s was used to disrupt the particles completely for accurate measurement of "free-GFP" molecules.

3.9 DIR-LABELLING OF EVS

EVs were generated as normally, however before the first UC spin or UF step, EVs were incubated with 1 μM DiR dye. After the incubation, the EVs were purified by UC or UF-LC. For certain experiments the DiR labeled EVs were then loaded onto a sucrose gradient to measure the density of the labeled vesicles and to make sure that there was no free DiR remaining after the washing of the EVs. The reason to use DiR for labeling the EVs is because DiR fluoresces near infrared and, hence the penetration *in vivo* is favourable. DiR also has a low background fluorescence when it is free and becomes highly fluorescent when bound to a lipid membrane.

3.10 *IN VIVO* INJECTIONS OF EVS

EVs were purified by UC or UF-LC and in certain experiments labelled with DiR. The EVs were then injected IV in the tail vein, subcutaneously (SC) or intraperitoneally (IP). When DiR labelled EVs were used, the biodistribution of EVs was analysed by the In Vivo Imaging System (IVIS), where both whole animal scans and scans of harvested organs were performed. In the initial dose comparison studies, a range of different EV doses was used. Subsequently, we used a fixed dose of 1.0 x 10^{10} particles/gram of animal body weight.

4 RESULTS & DISCUSSION

4.1 PAPER I

In this paper we have compared UC with ultrafiltration (UF) with subsequent size exclusion LC (UF-LC) for the purification of EVs. This study was undertaken because our lab struggled with low yields after UC purification and assumed (as many others in the field) that the high-speed ultracentrifugation harmed the EVs. In the article, EVs were carefully characterised to dissect the advantages and disadvantages with the two purification techniques. We found that the UF-LC purified biophysically intact EVs, with high yield and with a proteome that was highly similar to UC purified EVs. On the other hand, UC caused EVs to fuse, aggregate and/or break, and isolation yields were low and operator dependent.

Firstly, several UC protocols found in the literature were evaluated and we found that it was essential to wash the EVs with a second UC round to have as little contaminants as possible (results not included in the published article). Hence the UC protocol used for the comparison was similar to the one commonly used by the field that was presented by Thery *et al* in an article from 2006 (42).

Thereafter the particle amount was analysed after UC and UF purification respectively and according to NTA, UF produced significantly higher amounts of particles. However the apparent particle mode size remained the same between the two techniques despite the issues with vesicle aggregation and rupture when using UC. Furthermore, WB revealed stronger bands for UF samples if the same sample volume was loaded and the bands were of similar intensity when matched particle numbers according to NTA were investigated. However, when equal amount of protein was loaded onto the gel the UC purified sample displayed more intense staining for the same EV markers. Hence protein contamination was higher in the UF samples, the main contaminant being albumin as detected by LC-MS/MS.

We ventured on and investigated the biophysical properties of the vesicles by TEM and FCS to further study the vesicles after purification. Both TEM and FCS unearthed signs of aggregation and breakage of the vesicles after UC purification, whereas the vesicles after UF purification appeared to be more intact. In normal light microscopy CD63-eGFP labelled UC purified vesicles showed green punctate, whereas UF did not show any punctate at all, only diffuse background staining. Furthermore, total internal reflection fluorescence (TIRF) microscopy revealed a larger fraction of particles with an area exceeding 2 standard deviations of the mean size for UC samples, whereas UF-LC samples displayed a homogenous size distribution. Hence both light microscopy and TIRF corroborates the TEM and FCS finding that UC causes aggregation and/or vesicle fusion.

UF enriched significantly more particles that were intact compared to UC, however with high amounts of protein contaminants. Therefore we sought a method to further purify the vesicles with and selected size exclusion LC. After LC, EVs and albumin as well as other protein contaminants ended up in two different fractions and the EV samples were purer than UC purified samples according to the microgram protein per vesicle ratio (EV purity index) (50). Additionally, around 70% of the vesicles were consistently retained after LC purification and appeared intact according to TEM. The samples were also analysed by LC-MS/MS and the proteome of the EVs after either UC or UF-LC purification was highly similar, which was important since it showed that UF-LC purified the same vesicles as UC and the increased amounts of particles observed were indeed EVs and not protein contaminants. We also

showed that UF-LC could be extended to more heterogeneous/complex starting materials, such as stem cell media with maintained better purity as compared to UC.

Importantly, the biodistribution of DiR labelled EVs differed between the two purification techniques with UC purified EVs more readily taken up in lung compared to UF-LC samples. We speculate that EV accumulation in the lungs was most probably due to aggregation of EVs, which could be detrimental for therapeutic applications of such EVs.

In conclusion we showed that UF-LC retained more particles after purification that appeared intact compared to UC, which caused aggregation and breakage probably due to the high *g*-forces involved in the purification process.

4.2 PAPER II

The EL Andaloussi group devotes resources to develop EV therapeutics and, thus it is of great interest to understand which organs EVs reach upon systemic administration. Therefore, the biodistribution of EVs was investigated in Paper II. The DiR labelling technique was selected to track exogenously injected EVs in mice and we found that the dose, administration route as well as cell source influenced the biodistribution of EVs. Furthermore, by including a targeting moiety on the EV surface, uptake into brain was increased 2-fold. However, global biodistribution remained the same.

DiR labelling of EVs has been used frequently to track EVs *in vivo*, however little has been done to elucidate if free dye is co-purified with the EVs, which would influence the results and possibly lead to misleading conclusions. Hence we first characterised DiR labelled EVs after UC purification by floating the EVs on a sucrose gradient, which showed that free dye floats on a much lighter density than EV bound DiR and that no or insignificant amounts of free dye was co-purified with the labelled EVs, also suggesting sufficient labelling stability in a given time frame. The labelled EVs floated on a density similar to the density reported for exosomes and co-localised with the EV marker Alix according to WB. The morphology of labelled EVs was similar to unlabeled vesicles, appearing cup-shaped in TEM images. After the thorough characterisation, we were confident that the dye could be used to track labelled EVs *in vivo* and started with a dose escalation study to analyse how the dose would affect the distribution. The dose did affect the distribution to some degree with the highest dose accumulating less in the liver, possibly due to the saturation of the MPS. Hence, to avoid possible MPS saturating conditions, we chose the intermediate dose ($1x10^{10}$ particles/gram of body weight) for the following experiments.

It is not only the dose that can affect the fluorescence readings, since the dye has a long half-life, after a certain time the distribution pattern reflects the dye and not EVs. Therefore the distribution pattern at different time points (5 minutes to 48 hours post injection) was analysed. During the first 24 hours the distribution profile was similar, although at the 5-minute time point the accumulation in lungs was increased compared to later time points. At the 48-hour time point the distribution profile started to deviate from earlier readings with increased fluorescence in pancreas and declining values in liver. This could be due to re-distribution of EVs, a late phase uptake of remaining EVs or most probable an artefact due to the long half-life of the dye. Because of the risk of unspecific signals at the 48-hour time point, organs were harvested after 24 hours in the subsequent experiments.

The most common way to administer EVs according to the literature is by IV injections, while other injection routes are less studied, especially in comparative studies. Thus we chose to investigate if the administration method influenced the biodistribution of the EVs and injected DiR labelled EVs IV, SC and IP. Both SC and IP administered EVs accumulated less in the liver compared to IV injected EVs. Total tissue fluorescence was also weaker for SC administrated EVs when analysing individual organs *ex vivo*, possibly due to the retention of the EVs in adipose tissue. These results suggest that different injection locations may be utilised for different therapeutic targets, when different pharmacokinetics and/or biodistribution are desired.

Next, we examined whether EVs derived from cells originating from different tissues may possess organotropic properties. We included EVs from 3 mouse, 1 rat and 2 human cell types. The mouse cell sources were C2C12, a muscle cell line, B16F10, a melanoma cell line, and lastly primary bone marrow-derived immature DCs. The most significant observed difference was that DC derived EVs were distributed more efficiently to the spleen and less to the liver compared to the other cell type vesicles. The tissue distribution of EVs from rat and human origin was more similar to C2C12 and B16F10 than DC EVs. It is intriguing to speculate why DC-derived vesicles show this characteristic distribution. The repertoire of surface receptors and integrins has been shown to be important for EV uptake and distribution (described in more detail in section 1.7.2) and it is therefore likely that the DC-derived EVs possess a unique repertoire of surface molecules that allows the DC-EVs to engage more readily with the microenvironment in the spleen. The DCs were also the only primary mouse cell, as well as the only cell source of hematopoietic lineage, which may have an impact as well.

Lastly, the biodistribution of EVs engineered to be targeted to brain was compared to non-engineered EVs. Targeted EVs were derived from DCs that had been transfected with a chimeric construct of Lamp2b in fusion with the RVG-peptide that is derived from the rabies virus and have been shown to promote brain uptake of EVs (see section 1.10). The targeting moiety did not change the overall biodistribution as the highest signal was still originating from liver, spleen, gastrointestinal tract and lungs, however the accumulation in brain was 2-fold higher for the targeted EVs compared to non-targeted EVs. This highlights both the high sensitivity of the DiR labelling system in detecting subtle differences and that targeting moieties do not change the overall biodistribution, but may still allow for more accumulation in a targeted organ.

In conclusion we showed that the DiR labelling technique could be applied to EVs for *in vivo* tracking and biodistribution studies. Furthermore, the route of administration, cell source as well as targeting moieties influenced the biodistribution, although to different degrees.

4.3 PAPER III

Paper III is a manuscript that is a follow-up of Paper I although instead of SEC, a commercially available BE-SEC column was utilised for EV purification. We show that the BE-SEC column can be used to purify vesicles in a scalable, reproducible manner with yields of around 70% and still maintains superior purity compared to UC samples. Furthermore, the EV surface repertoire and uptake in recipient cells was similar between UC and BE-SEC samples.

The BE-SEC column has previously been used to purify viruses of similar sizes as EVs and hence it is already optimized for similar biological materials (208). The column consists of beads that allow material less than 700 kDa into the interior of the bead where it is trapped, while larger material is passing through the column without entering the porous resin bead. The hypothesis was therefore that the EVs would pass outside the beads and impurities in the sample would be confined inside the beads, according to the impurity binding capacity of the resin.

Firstly, we showed that the BE-SEC column could purify vesicles from non-concentrated cell culture supernatant. NTA, WB and TEM all indicated that the purified particles were indeed EVs. According to NTA, the method is reproducible both in terms of the yields achieved and vesicle sizes between samples. Hence, the BE-SEC column appears to be an attractive technology for EV purification.

Secondly the scalability of the technology was explored. Since the BE-SEC columns have a limit for the amount of protein they can bind, larger media volumes were first subjected to a diafiltration followed by a concentration step on a tangential flow filtration (TFF) device equipped with a 100 kDa molecular weight cut-off filter. The yield after TFF filtration was close to 100% and therefore the scalability of the process was further investigated. Consequently, increasing media volumes were purified with TFF/BE-SEC and according to NTA the increase of purified particles was reproducible and increased with increasing media volumes. Furthermore, did WB and TEM reveal vesicle markers and cup shaped particles respectively after TFF/BE-SEC purification. Hence, the BE-SEC method combined with a TFF step can be considered to be scalable.

The TFF apparatus can be fitted with filters of several different cut-off sizes. In an attempt to pre-purify the EV sample during the diafiltration step to a greater extent, we evaluated the performance of a TFF filter with a cut off size of 300 kDa. To our surprise the TFF 300 kDa filter retained over 95% of the EVs, contrary to the centrifugal filter units with the same molecular weight cut-off, which led to a significant loss of vesicles in Paper I experiments (unpublished data). Total protein staining and vesicles/μg protein both indicated that the 300 kDa TFF filter retained less protein impurities compared to the 100 kDa filter, which was expected. However the subsequent BE-SEC clean up step achieved practically the same final purity using either the 100 or 300 kDa filter cassettes for sample diafiltration, which for both was better than for a UC sample. However, since the EV recovery of the filters is the same, I believe the 300 kDa filter ought to be used for EV purification, since it does retain less protein contaminations and this can be important when larger media volumes would be used.

One major issue with EV purification is to separate the vesicles from non-vesicular material. To analyse BE-SEC performance in the latter, the TFF and TFF/BE-SEC samples were separated using an analytical Sepharose 4 Fast Flow SEC column. While the elution chromatogram of TFF samples displayed two peaks, the EV and non-vesicular protein and RNA peak, the TFF/BE-SEC samples showed only the EV peak. There can still be minor amounts of protein impurities left, however the levels were under the detection limit of both SEC and total protein staining. In the case of SEC, the protein impurities can also be of the same size as the EVs and therefore elute in the same peak, however if the protein aggregates would be that large they would probably be visible in the total protein staining, however this was not the case.

As has been described earlier, the protein surface repertoire of the EVs can be important for the biodistribution and therapeutic effect. Therefore, the surface proteins of TFF/BE-SEC and UC purified EVs were assessed by flow cytometry beads (34) in order to further verify that the TFF/BE-SEC is not biased in purifying only a subclass of vesicles. The bead assay revealed the same surface proteins with similar signal intensities for all positive markers for both samples. Hence the EVs purified with the two methods appear to carry the same surface proteins, which is very encouraging, especially when considering the shorter sample processing time of BE-SEC, which is important for achieving higher throughput.

Lastly, the uptake of CD63-eGFP labelled EVs in recipient cells was analysed by flow cytometer. When an equal dose of GFP positive particles was added to recipient cells the ΔMFI values were similar for TFF/BE-SEC and UC samples, indicating similar uptake characteristics. The uptake was dose dependent for both samples. However, the overlaid histograms of the GFP signal revealed a slightly more homogenous cellular uptake distribution for the TFF/BE-SEC sample, possibly related to a more homogenous EV sample compared to UC. In the article discussed in chapter 4.1 we showed that around 3% of the particles detected with TIRF microscopy in UC samples displayed a significantly larger area compared to the mean area size of the detected particles, whereas UF-LC purified samples were more homogenous with less than 0,5% of larger particles detected. Hence, the more heterogeneous histograms for UC purified samples can be due to aggregates in the samples.

In conclusion we show that the BE-SEC column can be utilised for EV purification and similarly to the UF-LC method (Paper I), it has unique advantages. It is scalable, reproducible with a high yield and better purity than UC purified samples. The BE-SEC columns can in the future be used as a last clean up step in the purification process of EVs for animal experiments and possibly even clinical samples for diagnostic purposes, however this remains to be tested.

5 FUTURE PERSPECTIVES

There are still several issues that are being debated in the EV community, such as a universal purification method, sample normalisation strategies, analytical techniques, storage conditions, buffers used and EV nomenclature. However since the field is advancing in such a rapid pace and new methods for characterisation and detection of EVs are published almost on a monthly basis (I have probably missed to mention a few in this thesis), I believe the field will homogenise in regard to these aspects.

In the EL Andaloussi group we work on a daily basis to develop EV based therapeutics and therefore we have invested many hours on sustainable purification methods, as presented in this thesis. Now, when we have established and thoroughly characterised purification methods that can be used for large media volumes, the aim is to further develop engineered EVs for targeted delivery of therapeutics. The latter requires overcoming many additional hurdles, such as short circulation time, possible undesired immune responses and better loading strategies for therapeutic molecules. In Paper II we show that EV biodistribution can be affected by changing the surface components of the vesicles, hence we are optimistic that other modifications may lead to increased EV half-life in blood and targeting to a tissue of interest, which is an important prerequisite for many therapeutic applications of EVs as envisioned today. EVs have certain features that make them interesting from a drug development perspective. They are natural conveyors of intercellular messages, are rapidly and effectively taken up by recipient cells, are relatively easy to engineer, they can be loaded with both therapeutically active RNAs and proteins and possess desired innate capabilities, which can be further enhanced by engineering approaches. However, care must be taken because of their general properties, also non-targeted cells are likely to interact with EVs and as described in chapter 1.9.2 EVs can aid in disease progression. Consequently, before entering clinical trails EVs from the cell source utilised must be carefully characterised and investigated in animal models. Furthermore, the loading efficiency of engineered EVs is still relatively low, especially for RNA therapeutics and an increase would allow for dose reductions and decreased risk of adverse events. Nevertheless, the effects achieved in animal models are pronounced after treatment with engineered/loaded EVs, which further indicate the great potential of EV therapeutics and EVs ability to convey their cargo to recipient cells.

Another on-going interesting research area is EV subpopulations and EV heterogeneity. Today not that much is known regarding subpopulations within the exosome and microvesicle field. Recent results indicating that subpopulation of EVs have different effects in *in vitro* models. This is very intriguing and may create further problems regarding both purification and classification of EVs in the future. Nevertheless if only a subset of EVs are responsible for a therapeutic effect the potential dose may be lower if only the effective subset could be purified. For engineered EVs this could be done already today with for example affinity chromatography columns.

It is too early to discern if therapeutically active EVs will ever reach the clinic outside clinical trails. However if not as a drug, EVs will probably within not such a distant future enter the clinic as biomarkers for certain diseases, such as various cancers. EVs utilised as biomarkers have not been covered in much detail in this thesis, it is, however an intense research field that is more mature than the EV therapy field. Hence, EVs will most probably be used in the clinical arena within the coming 10 years, hopefully both as therapeutic agents as well as biomarkers for disease.

6 CONFLICT OF INTEREST

I am a shareholder and consultant for EvOx Therapeutics Ltd.

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