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PROFILING AND EXPLOITING LIPID-BASED NANOPARTICLES IN VITRO AND IN VIVO

Helena Sork

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Profiling and exploiting lipid-based nanoparticles *in vitro* and *in vivo*

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Helena Sork

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

Co-supervisor:
Professor C.I.Edvard Smith
Karolinska Institutet
Department of Laboratory Medicine
Clinical Research Centre

Opponent:
Assistant Professor Dirk Michiel Pegtel
Vrije Universiteit Amsterdam
Department of Pathology
VU University Medical Center

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

Professor Gunnar Ronquist
Uppsala University
Department of Medical Sciences
Division of Clinical Chemistry

Minna Kaikkonen-Määtä
University of Eastern Finland
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences

To my kids, family and friends

Educating the mind without educating the heart is no education at all…

- Aristotle -

ABSTRACT

One of the major hurdles for therapeutic applications is the efficient delivery of bioactive molecules to the site of action. The high flexibility and biosafety of lipid-based nanoparticles has greatly enhanced their employment as delivery systems not only for synthetic but also for natural molecules such as proteins and nucleic acids. This thesis was brought about to investigate the nucleic acid delivery potential of synthetic lipid-based nanoparticles as well as to look into the composition and delivery patterns of their natural counterparts, extracellular vesicles (EVs), in order to set ground for future lipid-based therapeutic interventions.

Firstly, in Paper I we explored the potency of a lipid-based delivery agent, Lipofectamine 2000 which after being frozen and thawed showed orders of magnitude higher nucleic acid delivery efficiency *in vitro* and *in vivo* than the non-frozen counterpart. This effect was consistent across different cryo-manipulations, cell lines and also various types of nucleic acid. Further analysis with different methodologies revealed that the underlying potency plausibly relies on the elevated sedimentation and spreading of the complexes and/or relates to the specific structure or composition of the carrier. These findings illustrate that a simple freeze-thawing procedure allows to drastically reduce the amount of transfection reagent for cellular nucleic acid delivery, whilst not losing the desired activity.

Secondly, we shifted our focus to natural lipid-based carriers, EVs in order to shed light on the vesicular and non-vesicular (non-EV) small RNA patterns and their relation to the EV proteome (Paper II and III). Though the studies exploited different EV enrichment methods the relative depletion of vesicular small RNAs was confirmed in both instances. A detailed analysis of the secretory repertoire of small RNAs showed a significant depletion of microRNA (miRNA) sequences, matching well with the depletion of "miRNA related" proteins in EVs. The relative expression level of cellular, EV and non-EV miRNAs correlated well and though some differentially expressed (DE) miRNAs were detected, these had a relatively low expression in both the source cells as well as in the secretory fractions. We also quantified the total level of selected miRNAs in EVs and non-EV fraction investigating both the basal as well as overexpressed levels and could verify that the vast majority of mature miRNA is secreted to the non-EV portion of the secretome.

Paper IV was brought about to gain a comprehensive overview of the biodistribution of exogenous EVs. This study confirmed that fluorescent lipophilic dyes are suitable for membrane labelling and *in vivo* tracking of EVs. The general biodistribution pattern of EVs was seen to follow a common mononuclear phagocytic system (MPS) uptake pattern with the majority of EVs accumulating in the liver, spleen and lungs. Nevertheless, depending on the cell source, administration route, dose and the presence of targeting moieties this distribution could be altered.

The present findings are important to gain a thorough understanding of the nucleic acid delivery capacity of lipid-based nanoparticles, especially EVs and thereby progress their employment as therapeutic nucleic acid carriers.

LIST OF SCIENTIFIC PAPERS

I. **Sork H**, Nordin JZ, Turunen JJ, Wiklander OPB, Bestas B, Zaghloul EM, Margus H, Padari K, Duru AD, Corso G, Bost J, Vader P, Pooga M, Smith CIE, Wood MJA, Schiffelers RM, Hällbrink M, EL Andaloussi S. **Lipid-based Transfection Reagents Exhibit Cryo-induced Increase in Transfection Efficiency**. *Molecular Therapy Nucleic Acids.* 2016 Mar; 5(3): e290.

II. **Sork H**, Corso G, Krjutskov K, Johansson HJ, Nordin JZ, Wiklander OPB, Lee YXF, Orzechowski Westholm J, Lehtiö J, Wood MJA, Mäger I, EL Andaloussi S. **Heterogeneity and interplay of the extracellular vesicle small RNA transcriptome and proteome**. *Unpublished Manuscript*.

III. **Sork H**, Conceicao M, Corso C, Nordin JZ, Lee YXF, Krjutskov K, Orzechowski Westholm J, Vader P, Wood MJA, EL Andaloussi S, Mäger I. **Profiling the vesicular and non-vesicular miRNA secretome**. *Unpublished Manuscript*.

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

LIST OF ADDITIONAL PUBLICATIONS

I. EL Andaloussi S, Lehto T, Mäger I, Rosenthal-Aizman K, Oprea II, Simonson OE, **Sork H**, Ezzat K, Copolovici D-M, Kurrikoff K, Viola JR, Zaghlou EM, Sillard R, Johansson HJ, Hassane FS, Guterstam P, Suhorutsenko J, Moreno P MD, Oskolkov N, Hälldin J, Tedebark U, Metspalu A, Lebleu B, Lehtiö J, Smith CIE, Langel Ü. **Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo**. *Nucleic Acids Research.* 2011, 39(9), 3972 – 3987.

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III. Krjutškov K, Viltrop T, Palta P, Metspalu E, Tamm E, Suvi S, Sak K, Merilo A, **Sork H**, Teek R, Nikopensius T, Kivisild T, Metspalu A. **Evaluation of the 124-plex SNP typing microarray for forensic testing**. *Forensic Science International: Genetics.* 2009, 4(1), 43-48.

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CONTENTS

LIST OF ABBREVIATIONS

AFM	Atomic force microscopy
AGO 2	Argonaute 2
ALIX	ALG-2-interacting protein X
APC	Antigen presenting cell
ARF6	Adenosine diphospate-ribosylation ribosylation factor 6
ARRDC1	Arrestin domain-containing protein 1
CM	Conditioned medium
Ct	Cycle threshold
FBS	Fetal bovine serum
DC	Dendritic cell
DC-Chol	3β[N-(N′, N′-dimethylaminoethane)-carbamoyl] cholesterol
DE	Differential expression
DEAE	Diethylaminoethyl
Dir	1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide
DOPC	Dioleoylphosphocholine
DOPE	1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine
DOTAP	1,2-bis (oleoyloxy)-3-(trimethylammonio)propane
DOTMA	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride
DTT	Dithiotreitol
E	Efficiency (RT-qPCR)
EGFR	Epidermal growth factor receptor
ER	Endoplasmatic reticulum
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicle
Fgf21	Fibroblast growth factor 21
GO	Gene Ontology
GvHD	Graft-versus host disease
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
hnRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1
HRS	Hepatocyte growth factor-regulated tyrosine kinase substrate
HS	Heparin sulfate
HSP	Heat shock protein
HSPG	Heparin sulphate proteoglycan
ILV	Intraluminal vesicle
IL-10	Interleukin-10
i.m.	Intramuscular
i.p.	Intraperitoneal
ISEV	International Society for Extracellular Vesicles
i.v.	Intravenous

LBPA	Lysobisphosphatidic acid
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LF2000	Lipofectamine 2000
lncRNA	Long noncoding RNA
LNP	Lipid nanoparticle
miRNA	Micro RNA
MPS	Mononuclear phagocytic system
mRNA	Messenger RNA
mtRNA	Mitochondrial RNA
MV	Microvesicle
MVB	Multivesicular body
MWCO	Molecular weight cut-off
NSUN2	tRNA (cytosine(34)-C(5))-methyltransferase
NTA	Nanoparticle tracking analysis
OMV	Outer membrane vesicle
PANTHER	Protein ANalysis THrough Evolutionary Relationships
PBS	Phosphate buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PEG	Polyethylene glycol
piRNA	Piwi-like RNA
PLD2	Phospholipase D2
PS	Phosphatidylserine
RIPA	Radioimmunoprecipitation assay
RISC	RNA-induced silencing complex
rRNA	Ribosomal RNA
RT-qPCR	Quantitative reverse transcription PCR
RVG	Rabies virus glycoprotein
s.c.	Subcutaneous
SEC	Size exclusion chromatography
siRNA	Small interfering RNA
SDS	Sodium dodecyl sulfate
SR	Scavenger receptor
STAM1	Signal transducing adapter molecule 1
TEM	Transmission electron microscopy
tEV	Tumor-derived EV
TFF	Tangential flow filtration
TIRF	Total internal reflection fluorescence
TLR	Toll-like receptor
TNRC6A	Trinucleotide repeat-containing gene 6A protein
tRNA	Transfer RNA
TRPS	Tunable resistive pulse sensing
TSG101	Tumor susceptibility gene 101

UC	Ultracentrifugation
Uq	Ubiquitinylation
VT-RNA	Vault RNA
WB	Western blot
Wnt	Wingless
YB-1	Y-box binding protein 1

INTRODUCTION

The first reports describing the utility of nucleic acids in modulating gene expression were published in late 1970s [1,2]. Though the range and function of nucleic acids was at first rather narrow, a remarkable progress in the discovery of new RNA species together with their novel molecular functions has now been made [3–8]. This in turn has urged the development of a series of natural or chemically modified nucleic acid molecules, which can be harnessed as stand-alone therapeutic entities. Yet, the size and negative charge of nucleic acids hinders their passage through the cell membrane, implying to the need of advanced carrier systems tailored for their delivery into cells.

Delivery systems for nucleic acids can broadly be divided into viral and non-viral. The latter hold several advantages over viral systems including a higher level of biosafety and an improved flexibility to desired modifications. These conditions are met by non-viral lipid-based delivery systems, many of which are based on natural phospholipids providing a good biocompatibility, biodegradability and low immunogenicity compared to viral systems. The first generation of lipid-based nucleic acid delivery systems used either anionic or neutral lipids, which however were difficult and time consuming to produce [9]. Hence the interest was turned towards their cationic versions, which were found to significantly enhance the stability of the nucleic acid material as well as, owing to their positive charge, provide an improved interaction with the cell membrane [10].

Cationic lipids are often mixed with negatively charged nucleic acids to obtain delivery complexes known as lipoplexes. Yet, they are more frequently employed for creating structured lipoplexes, such as liposomes [11]. To date, a myriad of different cationic lipid-based transfection reagents have been developed mainly for *in vitro* screening purposes. The discovery of Lipofectin [12] greatly enhanced the use of cationic lipids for nucleic acid delivery and ignited the studies of lipid-based nanocarriers also for human gene therapy applications [13].

In addition to synthetic lipid-based nucleic acid delivery systems, recently an increasing interest has been paid to nucleic acid delivery properties of naturally occurring membrane-enclosed vesicles, called extracellular vesicles (EVs). Though earlier reports of plasma membrane derived vesicles exist [14], the first studies on vesicles of endosomal origin were published in 1980s [15–17]. Initially, they were thought to purely represent ‘garbage bins’ to discard cellular waste [15]. However, a decade later evidence of EV mediated signalling was presented [18], opening a brand new research field of intercellular communication. During the years thereafter the EV biology field has witnessed an explosive growth, constantly revealing new pivotal roles of EVs, disclosing their potential as disease biomarkers as well as natural nanoparticles for therapeutic macromolecule delivery.

This literature review will give a brief overview of lipid-based nucleic acid delivery systems, by briefly covering synthetic cationic lipid-based delivery vectors and having an emphasis on their natural counterparts, EVs.

1 Synthetic cationic lipid-based delivery systems

1.1 General overview and composition

The size and negative charge of DNA and RNA hinder their passage through the plasma membrane and thereby precludes their naked usage as stand-alone therapeutic compounds. Moreover, nucleic acid polymers are susceptible to nucleases present in the biological fluids, causing their degradation within minutes [19–21]. Shorter nucleic acid stretches are also subject to rapid clearance by renal filtration, significantly decreasing their bioavailability in target sites where they additionally face plasma-, endosomal and nuclear membrane barriers [22]. As systemic administration of unmodified nucleic acid can furthermore stimulate Toll-like receptors (TLRs) and thereby activate innate immune response [23], sophisticated delivery vehicles that would enhance the bioavailability of nucleic acids are needed.

The use of cationic lipids for nucleic acid delivery was first described by Felgner *et al.* in 1987 [12], reporting a log scale improvement in transfection efficiency as compared to conventionally used calcium phosphate or diethylaminoethyl (DEAE)-dextran mediated gene delivery. The general complexation rationale of cationic lipid mediated delivery relies on the formation of electrostatic interactions between the positively charged hydrophilic head group of the lipid and negatively charged phosphate group of the nucleic acid, whereby neutralizing or increasing the overall charge of the complex to aid cellular delivery. Depending on the complexation parameters, cationic lipids form highly structured entities or complexes with irregular morphology, referred to as liposomes and lipoplexes, respectively [24,25]. In the lipoplex, the complexed material is partially condensed and their structure does not resemble that of liposomes. Instead, the lipoplex appears as a multilamellar liquid crystal consisting of hydrated DNA layers alternating with cationic lipid bilayers [24]. Also, other molecular configurations, such as columnar hexagonal phase structures have been described [26]. Though the dynamics of lipoplex assembly are poorly understood, it has been shown that lipid packaging parameters dictate the organization of the structure [27]. Schematic illustration of a lipoplex and a liposome are depicted on Figure 1.

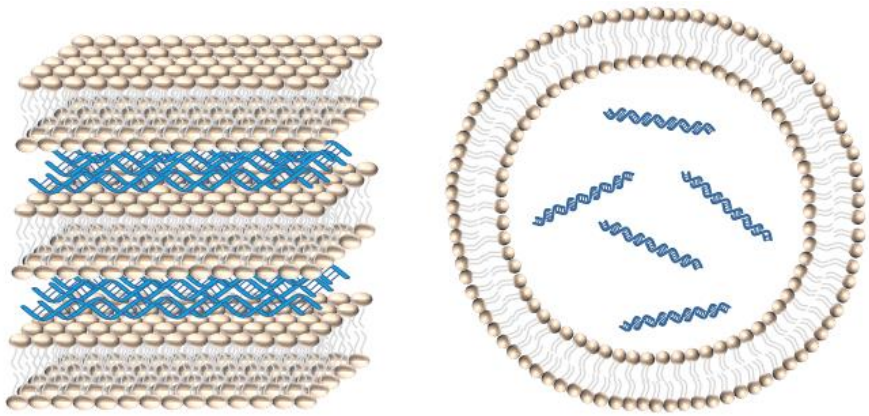

Figure 1 - Illustration of a multilamellar lipoplex (left) and a spherical liposome (right) encapsulating nucleic acid.

The first discovery and description of liposomal structures dates back more than 50 years when Bangham and colleagues described "bangosomes" [28]. These artificial spherules rapidly gained interest and a few years later received a more descriptive term "liposomes" [29]. Liposomes are spherical vesicles of 20 nm to a few micrometers in diameter that are composed of one or multiple phospholipid bilayers, entrapping a solute of interest. Unlike lipoplexes, which most often have an irregular structure, the lipids in liposomes are organized in a spherical manner, providing a capsule for the therapeutic moiety. The lipid core protects the content from degradation, decreases its cellular toxicity as well as enhances its solubility and stability *in vivo* [30]. In addition, owing to their lipid architecture, liposomes are amenable to surface engineering, being beneficiary not only to enhance their uptake, but also to introduce specific ligands for improved tissue targeting [31,32].

In addition to the aforementioned, cationic lipids are also increasingly used to form lipid nanoparticles (LNPs), both for academic studies as well as clinical applications [33,34]. LNPs have a similar composition as liposomes, constituting of cationic lipids and helper lipids, yet often have a solid, lipophilic core region. The encapsulation of nucleic acids into LNPs is usually performed by microfluidic mixing, generating lipid nanostructures containing inner inverted micelles of cationic lipids complexed with the nucleic acids [35]. Owing to the highly efficient nucleic acid encapsulation, providing the formulation a good potency [36], LNPs are one of the most widely used and efficient gene delivery vehicles both for *in vitro* as well as *in vivo* applications. Moreover, as these nanoparticles allow to simultaneously exploit the therapeutic benefits of nucleic acids and encapsulated lipophilic drugs [37], they are also at lead among all non-viral delivery vehicles that are used for clinical purposes [34,38].

The lipids that are used to form the aforementioned structures can be anionic, neutral or cationic. Considering the polyanionic nature of nucleic acids, the use of anionic lipids has rather been directed towards the delivery of other therapeutic macromolecules [39]. Well-characterized and widely used cationic lipids for cellular delivery purposes include [1,2-bis (oleoyloxy)-3-(trimethylammonio)propane] (DOTAP) [40], N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) [12] and 3β[N-(N′, N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) [41]. Occasionally, these lipids are used as solitary nucleic acid carriers, although most often they are mixed with neutral helper-lipids (e.g. 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE), dioleoylphosphocholine (DOPC) and cholesterol) [12,42] to enhance membrane fusion and aid endosomal escape[42]. Though the basic structure of these lipids mimics chemical and physical attributes of biological lipids, structural differences in the size of the head group and length of the hydrocarbon tail give distinct characteristics to the complex, affecting cellular association and uptake.

1.2 Uptake and cargo release

For the complexes to be efficiently taken up by the cells, a high extent of condensation as well as a net positive charge is preferred. The interaction of anionic liposomes with cell membrane is somewhat unclear, however it has been demonstrated that the uptake will take place once a certain threshold of cell-to-liposome charge ratio is exceeded [43]. Cationic complexes are known to electrostatically interact with the negatively charged glycoproteins and proteoglycans on the cell membrane, thereby facilitating cellular interaction and membrane transversal via endocytosis [44,45].

It is known that larger lipoplexes enter the cells more easily than smaller ones due to their improved sedimentation [46], facilitated membrane contact/fusion and easier dissociation of the complex after successful endocytosis [47,48]. By increasing the cationic lipid nucleic acid ratio, larger lipoplexes with a higher positive net charge can be created. Yet, too high concentration of lipids interferes with the functions of the cellular and subcellular membranes, thereby compromising cellular integrity and causing cytotoxicity [49]. In addition, even at non-cytotoxic concentrations, the surface charge of the complexes itself can cause genotoxic events and lead to the significant formation of micronuclei in cells [50,51].

In order to result in successful nucleic acid delivery, the complexes face a rate-limiting step of endosomal escape and cargo release from the lipid material. Firstly, it must be noted that the majority of the endocytosed material gets targeted to lysosomal degradation [52,53], the remainder needs to cause a transient destabilization of the bilayered lipid structure of the endosome and reach the site-of-action. The cargo release is often promoted by the incorporation of helper-lipids, which not only facilitate interaction with the cell membrane but owing to their fusogenic properties also mediate endosomal escape [42,54,55]. In addition, it has been hypothesized that the buffering capacity of nanoparticles prevents acidification of endosomal vesicles, thereby activating the influx of protons and counterions, causing osmotic swelling and rupture of the endosome and cargo release into the cytoplasm. However, the so-called “proton sponge” effect is still a matter of active debate [56]. The dissociation of nucleic acid from cationic lipoplexes in not fully understood, though it has been suggested that charge neutralization and structural changes (phase evolution of lipoplex lipids) induced by the interaction with cellular anionic lipids are decisive for a successful outcome [57–59].

Larger lipoplexes are generally only beneficial for *in vitro* settings, as an improved *in vivo* delivery is commonly obtained with small particles being less prone to clearance by the MPS responsible for the elimination of foreign material [60]. Also, the positive surface charge, being beneficiary for improved cellular delivery, could lead to a reduction in circulation half-life and thereby inversely affect the transfection efficiency *in vivo* [61,62]. This is partly attributed to aggregation of the complexes with negatively charged serum proteins as well as hemagglutination with erythrocytes [63] leading to their fast clearance from the circulation and limiting their usage beyond vascular endothelial cells [64]. To avoid these processes, the surface of liposomes and LNPs is often shielded by polymers, such as polyethylene glycol

(PEG) or glycolipids (e.g. gangliosides) which provide a neutral hydrophilic layer avoiding vesicle aggregation, thereby increasing the circulation time and decreasing immune response *in vivo* [65–68]. Formation of this hydrating layer by the association of the ethylene glycol units of PEG and water molecules hinders protein adsorption and subsequent clearance of the complexes by the MPS, providing the complexes a so-called "stealth" behaviour [67,69].

1.3 Commercial lipid-based delivery agents

Owing to the rapid, scalable and highly reproducible manufacturing process, long shelf-life and ease of handling, commercial lipid based reagents have proved themselves as robust means of gene delivery, especially for *in vitro* screening purposes. Their applicability has prospered largely owing to the accompanying development of assay-based reporter gene systems providing a quick and easy readout to the biological questions of interest.

The pioneering commercial reagents consisted of well-known lipids (e.g. DOTAP, DOTMA, DC-Chol), exhibited rather good transfection efficiencies in different cell models, yet were relatively ineffective for *in vivo* applications (reviewed in [70]). One of the earliest and most widely used transfection reagent to date is Lipofectin [12]. This proprietary blend of DOTMA/DOPE at 1:1 molar ratio was launched in late 1980s and is considered as one of the first lipid-based transfection reagents, which boosted the employment of lipid-based nanocarriers not only for *in vitro* but also for *in vivo* purposes. Lipid mixtures have thereafter been constantly improved, by modifying the head group size or hydrocarbon tail length, giving distinct characteristics to the lipoplex regarding association and uptake into the cell. To date, a series of transfection reagents have been launched, tailored for the transfection of specific types of nucleic acids or cells, formulated for improved gene delivery *in vivo* or serving as broad spectrum delivery agents. The majority of them are proprietary lipid-based blends, which can form complexes with the nucleic acid via a simple co-incubation. Since commercial lipid mediated transfection gives highly reproducible results and high transfection efficiency [71], initial screening purposes are now devoid of the need for specific instruments and specialized personnel hampering the wide-spread employment of earlier lipid formulations.

The choice of a commercial reagent largely depends on the delivered macromolecule (DNA, RNA or protein) and the type of cell that needs to be transfected (adherent, suspension, primary). The vast majority of commercial reagents have been developed for the delivery of exogenous nucleic acids, with a range of distinct formulations fine-tuned for each of their subtypes (plasmids, oligonucleotides, messenger RNA (RNA), small interfering RNA (siRNA) etc.). Yet, broad spectrum transfection reagents (e.g. Lipofectamine 2000 (LF2000)) in terms of cell- and macromolecule type are generally more popular, excluding the need to handle diverse protocols.

Owing to the vast number of commercial lipid-based reagents available, detailed dissection of their types and properties is out of the scope of this review. The choice of the reagent depends

on the research question and specific experiments in mind. Depending on the required transfection efficiency or sensitivity of the downstream analysis, screening of many products for optimal results might be required. Nevertheless, due to the high cost, such experiments are mostly limited to small-scale *in vitro* screenings, leaving extensive *in vitro* as well as *in vivo* studies still relying on the usage of custom-made formulations.

2 Natural delivery vectors - extracellular vesicles

Despite the vast developmental efforts, the transfection efficiency of synthetic lipid-based systems still succumbs to natural carriers. Though synthetic vectors commonly need novel interventions to overcome cellular barriers, natural delivery systems have already developed several of these merits during the course of their evolution.

EVs are known for their native ability to cross biological barriers. Owing to their high potency to enter cells together with the susceptibility to membrane- or content modifications, EVs have drawn increasing attention as natural delivery vectors for different biologics. The first report, later acknowledged as describing EVs, outlined the isolation of a "clotting factor" in plasma which pelleted at high-speed centrifugation and modulated the clotting time [72]. Yet, it took more than two decades until additional studies on cell-derived vesicles were published [14,73,74]. At first, these vesicles were collectively referred to as "exosomes", a term initially proposed by Trams *et al.* in 1981 [75]. Yet, at the time the subcellular origin of these vesicles was still unclear. The endolysosomal EV biogenesis pathway was elucidated a couple of years later when two groups, investigating the recycling of transferrin receptor, demonstrated the release of EVs from multivesicular bodies (MVBs) in two papers published only a week apart from each other [15,76]. Thereafter, the term "exosomes" was revived [17] and gained a wider acceptance in the scientific community. Nevertheless, EV research was largely neglected until 1996 when Raposo *et al.* [18] described the communication between B cell-derived EVs and T cells, indicating to the functional role of EVs in cell-to-cell communication. The next big milestones in EV-research denote the discoveries of EV-mediated transfer of functional RNA between cells [77,78] as well as across the blood-brain-barrier [79]. The latter further escalated the interest in EVs and opened novel avenues for their employment as therapeutic nucleic acid carriers.

2.1 EV terminology and classification

EVs have attracted interest in disparate research fields, which on one hand has allowed a quick reveal of a myriad of biological roles, on the other hand, led to a "stretched" terminology as well as lack of standardization and consensus among researchers [80].

Several different names for EVs have been proposed, relating them to their specific functions (e.g. "tolerosomes" that induce immunological tolerance to dietary antigens [81] or "oncosomes" [82] shedded by tumours), or the cell of origin like "prostasomes" [83] (prostate epithelial cell vesicles) and "epididymosomes" (deriving from epididymal epithelial cells) [84]. As these are only suitable within specialized research fields, commonly a definition based on biogenesis, distinguishing between exosomes, microvesicles (MVs) and apoptotic bodies, is used. Exosomes are referred to as vesicles that are generated via intraluminal budding of the early endosome and secretion to the extracellular space by the fusion of the MVB with the plasma membrane. Microvesicles, on the contrary, are mostly referred to as

vesicles that directly bud from the cell membrane [85]. In addition to biogenesis, these subtypes also differ in size, exosomes ranging from 30-120 nm and microvesicles typically between 100-1000 nm [86]. Moreover, also apoptotic bodies are considered as one of the subgroup of EVs [87]. These vesicles are typically bigger than MVs (1000-5000 nm), originate from plasma membrane budding and/or endoplasmatic reticulum (ER) and often contain nuclear fragments [88–90].

In addition to aforementioned, also physical characteristics such as density and/or necessary pelleting force are used to distinguish the different EV classes [80,91–93]. Yet, all these approaches should rather be considered complementary to each other and demand additional biochemical analysis in order to provide a detailed characterization of the preparation.

Considering the disorder in the nomenclature, together with the evidence of size, density and surface marker overlap between the EV subgroups [94,95], it has now been widely acknowledged that secreted membrane vesicles should collectively be referred to as extracellular vesicles [80]. Furthermore, an increasing number of studies are characterizing different EV subpopulations with inherent physical characteristics, RNA content and protein repertoire [96–99], indicating to the need of an even more sophisticated nomenclature to cover the differences. All this contributes to confusion in data interpretation and comparison between studies, yet important steps towards an improvement in the nomenclature and experimental settings have been made [80,100]. Here, I use the generic term EV to refer to all cell-derived membrane enclosed vesicles (except for apoptotic bodies). Depending on the importance to the specific context, exosomes and MVs might be named separately.

2.2 EV isolation

EVs are most commonly purified from conditioned cell culture medium or from biological fluids such as urine, blood, cerebrospinal fluid or saliva with the composition of the material greatly outlining the obstacles and choice of EV isolation methodology. All these fluids are highly complex and in addition to EVs, also contain non-vesicular macromolecules (proteins, nucleic acids), cell debris as well as apoptotic- and lipoprotein particles. Hence, the isolation of EVs is technically challenging not only due to their size and heterogeneity but also due to the multifaceted nature of the surroundings.

2.2.1 Consideration of the starting material

For cell culture derived material, the biggest concern is the presence of “additional” vesicles, originating from apoptotic cells, fetal bovine serum (FBS) or arising from other media supplements. These co-purifying vesicles might mimic the effect of cell derived EVs and thereby pose a risk of introducing false positive results.

Cell debris and apoptotic particles are largely removed by sequential centrifugation steps at 300 × g and 2,000-3,000 × g, respectively. To minimize the number of FBS-derived particles, it is advised to propagate the cells either in serum free conditions or in EV-depleted FBS

[101], through phenotypic changes of the source cells must also be considered [102]. Of note, in addition to particulate matter, FBS also contains a vast amount of proteins as well as diverse repertoire of RNA species, which cannot fully be eliminated and might lead to misinterpretation of the data [103,104]. Hence, even though the cell culture environment is rather defined, several different factors can affect the final outcome of the study, emphasizing the need of controlling and tailoring also *in vitro* conditions.

Biological fluids are even more complex than the defined environment of cell culture material, demanding individual approaches to yield EV preparations largely free from contaminating particulates, proteins and RNA. For example, in plasma, it has been estimated that as much as 95-99% of that extracellular RNA is not bound to EVs but rather to proteins (e.g. Argonaute 2 (AGO 2) [105]) and lipoprotein particles [106,107] representing potential co-isolates for EV preparations [108,109]. In addition, blood borne material is viscous and rich in "sticky" proteins such as albumin, posing further challenges to obtain a clean preparation and emphasizing the importance of tailored EV enrichment methods.

Below, a brief overview of the most widely applied EV isolation methods is provided with an emphasis on ultracentrifugation (UC) and size exclusion chromatography (SEC) owing to their employment in the constitutive papers.

2.2.2 Ultracentrifugation

To date, the method with the longest track record and widest application for EV isolation is UC. This involves a series of centrifugation steps, whereby the cell debris and apoptotic particles are first pelleted at 300 × g and 2,000-3,000 × g, respectively followed by an optional centrifugation at 10,000-20,000 × g to remove bigger EVs, often referred to as MVs. In order to further enrich for the presence of smaller vesicles the latter step can be substituted or followed by a sterile 0.22 μm filtration continued by UC at 100,000-120,000 × g to collect particles usually referred to as exosomes [110]. The purity of the preparation can further be enhanced by an additional wash step or density gradient separation that separates EVs based on their buoyant density and helps to additionally reduce protein contamination [111]. Yet, the process is time consuming, unsuitable for high-throughput analysis and is most practical as a follow-up method, since particles with a similar density to EVs cannot be discriminated [109].

Despite the wide employment of UC purification for cell culture derived- as well as biological material [112], recent evidence outlines a number of shortcomings (e.g. compromised purity, aggregation, decreased vesicle intactness) related to the methodology, resulting in a decreased functionality [113] and altered biodistribution *in vivo* [114]. Therefore, increasing efforts have been made to develop "milder" purification strategies with improved capacity to retain the biophysical properties of EVs.

2.2.3 Size exclusion based methods

The employment of SEC for EV purification is increasingly practiced and promoted by the EV community. The principle of SEC relies on the fractionation of particles by size, transiently trapping smaller molecules in porous beads, whereas larger ones bypass the matrix and elute earlier [115,116]. Owing to the lack of high *g* force and extended sample handing time, SEC results in improved integrity, purity and functionality of the vesicles compared to UC [113,117,118], is less operator dependent as well as shows consistent recovery rates across isolations [114]. In addition, SEC allows a fine fractionation of the whole secretome [119], remaining largely out of reach with centrifugation based approaches. Also, the method can be tailored by the type of gel matrix, pore size and column length to either perform routine vesicle preparations up until sophisticated studies on EV subpopulations [98,120]. As the pre-packed gravity flow columns are also commercially available, SEC has gained increasing popularity as a rapid EV isolation technique. Yet, the methodology is still somewhat constrained in its scalability, limiting its employment for large-scale *in vivo* studies as well as engagement in clinical applications. To overcome this drawback, SEC is often combined with filtration-based techniques [114], enabling the processing of large sample quantities while retaining the benefits of SEC regarding the yield, purity and biophysical properties of EVs.

Another size exclusion based techniques that has rapidly gained popularity is a commercially available tangential flow filtration (TFF) system. The TFF method is based on a cross-flow filtration process through a semi-permeable membrane filtration unit which has a fixed molecular weight cut-off (MWCO) limit. The pores allow the passage of small molecules through the membrane, while the larger ones are entrapped and remain in the circulation. Simultaneously with the size separation, the method allows to obtain a highly concentrated EV sample, being especially valuable for downstream applications such as SEC [121].

2.2.4 Alternative EV isolation approaches

In addition to centrifugation and size-based isolation methods a large variety of approaches making use of the molecular, biophysical or biochemical characteristics of EVs have been developed. Some utilize the surface markers of EVs, such as immunoaffinity capture-based techniques and have proven to be useful if only a subtype of EVs is aimed to be investigated [103,122]. Others employ polyanions, such as heparin to capture the full diversity of different EV types [123]. In addition, several magnetic isolation techniques [124] as well as a plethora of lab-on-chip devices have been developed [124,125], with newer approaches opening avenues for cancer diagnostics [126]. To prepare EV samples in a simple and quick manner, some methodologies take advantage of the change in EV solubility and employ hydrophilic polymer solutions such as PEG or commercial polymer-based preparations (e.g. ExoQuick™). As the purity of such isolates is rather poor [127], the usage of these methods beyond rapid EV assessment is rather limited. Yet, in clinical settings the purity and essence of the active component might be less important, permitting a successful employment of precipitation based protocols for EV therapeutics [128].

In conclusion, the complexity and variety of EV sources is the main driving force for the development of novel isolation methodologies. Biological samples are much more complex, heterogeneous and in limited quantities compared to cell-culture derived material, restraining the choice of potential purification methods and often resulting in a low amount of EVs. On the other hand, cell-culture preparations are more uniform, but need scalable isolation methods and might be influenced by culture additives which could skew the biological outcome. Hence, the type of purification method to use is highly dependent on the origin of the sample as well as the type and extent of downstream analysis.

2.3 EV characterization

The insufficient understanding of basic EV biology is a major limiting factor for describing the identity of the isolated vesicles. Therefore, the International Society for Extracellular Vesicles (ISEV) has released a number of position papers providing guidelines and describing the minimal experimental requirements for the definition of vesicular preparations [100,129,130]. These suggest the characterization of EV size and density, vesicle morphology as well as the analysis of EV-associated proteins as discussed hereafter.

2.3.1 Physical characterization

The most basic parameters for the characterization of EV preparations include the description of size and morphology of the vesicles. In the early studies, these characteristics were evaluated by transmission electron microscopy (TEM) [17], which is still the most widely used methodology for morphological characterization and is at times operated at cryogenic conditions to better preserve the native state of the material [131]. Newer microscopy techniques such as atomic force microscopy (AFM) are less used, yet also allow to measure the sample in their native condition, output a three dimensional topography of the material and are able to yield quantitative information on surface proteins [132].

Though microscopy techniques also enable the determination of particle size, the process of obtaining such data is rather tedious and limited in the number of representative events. Hence, several newer methodologies such as nanoparticle tracking analysis (NTA) [133] and tunable resistive pulse sensing (TRPS) [134] have been developed. However, the outcome of such technologies greatly relies on the EV isolation methods as contaminating particulate material (e.g. lipoproteins) cannot be discriminated from EVs.

In addition to size and morphology, it is advised that EVs are also characterized by their density, ranging around 1.13- 1.19 g/ml in sucrose or iodixanol gradients [135]. Owing to the capability to form iso-osmotic solutions at a wide range of densities together with the decreased sedimentation time for separation [135], iodixanol (commercially known as OptiPrep™) is increasingly employed for density based EV analysis. Yet, the lack of standardization of density gradient centrifugation protocols causes slightly variable results between different studies. In addition, the method does not allow for the distinction of certain

contaminants e.g. high-density lipoproteins from EVs [109], illustrating the importance of tailored EV isolation approaches.

2.3.2 Molecular characterization

In addition to physical parameters, EVs are known to possess a range of molecular signatures, mostly featuring characteristic enrichment patterns in certain proteins and lipids. Since the current knowledge of the EV proteome has yet not allowed to determine any EV-specific markers, the community rather adheres to experimental guidelines suggesting the verification of a relative abundance/depletion of different proteins [100]. To evaluate the composition of EV-associated proteins, easily accessible assays such as western blot (WB) and other immunoblot methods (e.g. dot-blot [136]) are commonly used. However, the mere probing for a selected set of EV markers is increasingly replaced with the employment of high-throughput proteomic technologies allowing a closer look on the overall EV protein content [99,114,137]. The detection of surface markers can also be achieved by flow cytometry approaches using multiplex bead-based platform, investigating tens of different surface markers simultaneously [122].

It is known that the EV membrane composition largely reflects that of its source cell. Yet, similarly to the enrichment of certain proteins, distinct lipids (e.g. sphingolipids and cholesterol) are still known to be overrepresented [138]. The relatively late discovery related to the biological activity of vesicular lipids [139] somewhat delayed the interest in the lipid composition of EVs. Presently, more studies employ chromatography and/or mass spectrometry based methods to perform solitary lipidomic or combined omics analysis on EVs [140,141] and constantly improve our understanding of the molecular composition and sorting of vesicular lipidome.

In addition to proteins and lipids, EVs are also, as aforementioned known to contain nucleic acids. The presence of vesicular DNA is largely disputed [129,142] and often considered as contaminants in the EV preparations. The EV RNA content is enriched in short molecules [143,144] which are either encapsulated in EVs or reside on their surface, where they are also considered as an isolation method-derived impurity. Hence, to ensure the reliability of downstream EV nucleic acid analysis (e.g. sequencing, microarrays, quantitative reverse transcription PCR (RT-qPCR)), enzymatic treatment of the preparations with DNase and/or RNase combined with detergent treatments is advised [130]. Nevertheless, considering that the relative quantity of a distinct nucleic acid molecule per EV is low and other co-isolates such as lipoprotein particles remain untouched by the enzymatic treatment, a reliable detection and quantification of EV RNA still remains a challenge. In addition, depending on the sample type, the EV preparations contain a combination of DNA and RNA molecules affecting the downstream analysis of one or the other. Though a few methods (e.g. Qubit HS RNA assay) efficiently discriminate between these nucleic acid types, others (e.g. Nanodrop, Agilent Bioanalyzer chips) measure a combination of both, being a subject to fluctuation and misinterpretation of the final data. Attention must also be turned to nucleic acid isolation

methods as well as deep sequencing protocols allowing a clean preparation of a single nucleic acid type, thereby avoiding the acquisition of mixed molecular signatures.

The small size, heterogeneity and our poor knowledge of EV biology make their characterization particularly challenging. The availability of public online databases has greatly expanded our understanding of the diversity of EV features. Yet size, morphology, buoyant density and surface marker detection are still most commonly investigated characteristics. Emerging new methods, among others measuring the optical- and surface charge properties or tracking single vesicles [145–147] underpin the progress in EV characterization with new exciting techniques yet to be unveiled.

2.4 EV biogenesis and secretion

The presence of "coagulant material in minute particulate form" originating from platelets was already described in 1967 [14], followed by the description of plasma membrane outward budding mechanism a few years later [148]. Based on the current knowledge, this biogenesis mechanism is rather attributed to MVs (and apoptotic bodies), whereas the classical exosome biogenesis differs considerably from this process and follows the routes of endolysosomal recycling pathway [80]. A schematic overview of EV biogenesis is depicted on Figure 2.

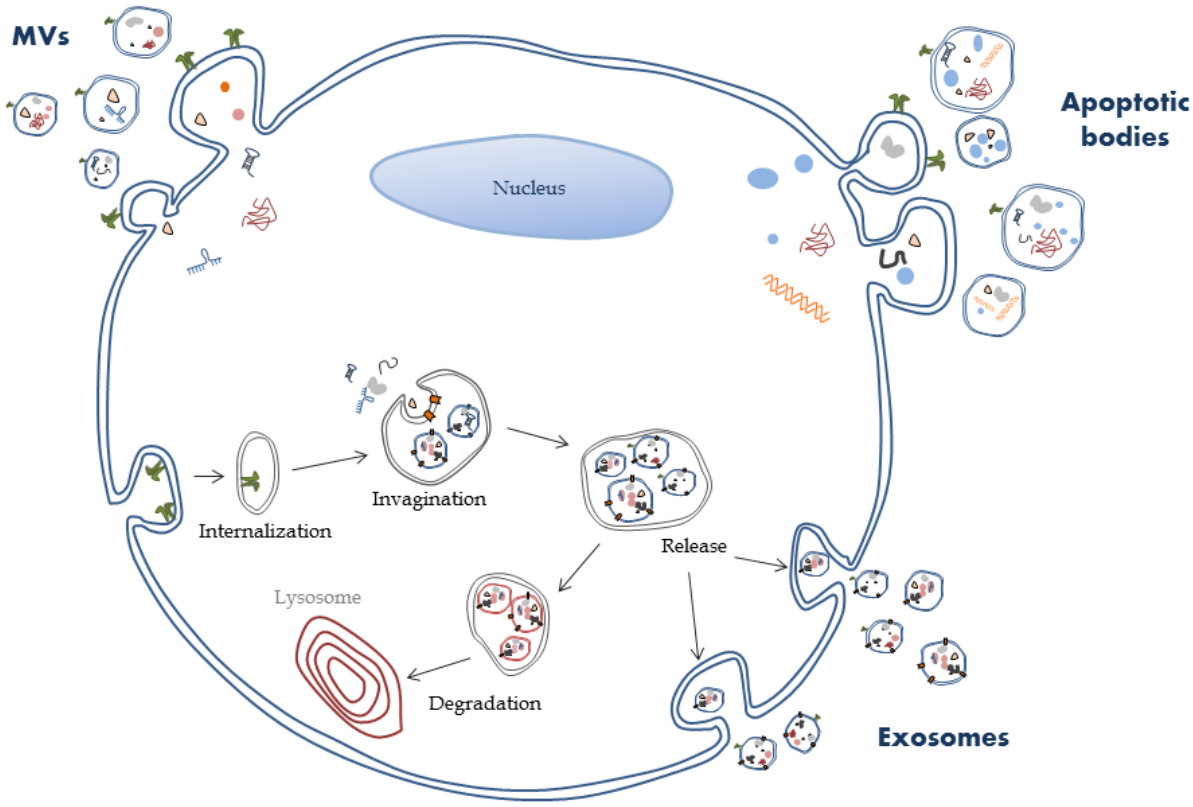

Figure 2 - Illustration of EV biogenesis.

2.4.1 EVs derived from the endolysosomal pathway

2.4.1.1 General overview of biogenesis

The endolysosomal pathway is known to consist of numerous membrane compartments responsible for the catabolism and recycling of material retrieved via extracellular endocytosis or intracellular autophagy [149,150]. This knowledge was later complemented with the observations of Dr. Rose Johnstone, describing the presence and release of vesicular structures via late endosomes and exposing a new role of the pathway in cellular biogenesis [15,17]. From then onwards, a number of studies have concentrated on understanding the degradation "escape" routes of the endolysosomal system and revealed a number of different components responsible for the biogenesis of endolysosomally-derived EVs, commonly referred to as exosomes.

The endocytosis of extracellular material results in the formation of early endosomes, which will either be recycled back to the plasma membrane, or mature to late endosomes [149,151]. The membrane invagination activity on the late endosome gives rise to the formation of intraluminal vesicles (ILVs), forming MVBs which either fuse with the lysosomes to degrade the content [150] or will head towards the periphery of the cell, merge with the cellular membrane and give rise to exosomes in the extracellular space [149].

2.4.1.2 Biogenesis pathways

For exosome biogenesis, mainly two types of pathways have been described: endosomal sorting complex required for transport (ESCRT) dependent [152] and -independent pathway [153]. The former starts with the initial clustering of ESCRT-0, -I and -II protein complexes at tetraspanins-enriched membrane microdomains [154]. These complexes sort ubiquitinylated proteins to the endosomal membrane and initiate the formation of intraluminal buds. With the involvement of ESCRT-III, the membrane abscission is completed resulting in the generation of MVBs containing tens of ILVs [155–157].

The involvement of the ESCRT machinery was first found through proteomic studies [158] and its role in exosome biogenesis has thereafter been extensively studied. One possible mechanism how ESCRT dependent MVB formation can occur was described in a study by Baietti *et al.* [152], outlining how the expression levels of ALG-2-interacting protein X (ALIX; ESCRT-III associated protein), syntenin as well as syndecan modulate ILV formation and subsequent exosome release. Later, it was revealed that this process is regulated by a small GTPase ARF6 (adenosine diphospate-ribosylation ribosylation factor 6) and phospholipase D2 (PLD2) [159] and is activated by heparanase, trimming the heparan sulfate chains on syndecans [160]. Studies have also shown a clear linkage between the ESCRT-0 protein hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) and exosome secretion [161,162]. Yet, the production of the aforementioned syntenin exosomes requires only certain ESCRT components but is independent of others [152]. Additionally, inactivation of four proteins representing different ESCRTs has been found not to inhibit the

formation of MVBs [163], suggesting the existence of an alternative, ESCRT-independent mechanisms. In the light of the aforementioned, a HRS competing, CD63-dependent formation of ILVs was reported [164], indicating to the possible coexistence of mixed EV populations in a single MVB. Additionally, higher order oligomerization of membrane proteins has been found sufficient for the formation of human immunodeficiency virus (HIV) Gag ILVs [165], a mechanism corroborating with data on syntenin exosomes [152], requiring syndecan clustering for their biogenesis.

It has also been observed that the lipid composition of exosomes is remarkably similar to that of lipid rafts, showing high enrichment in sphingolipids and cholesterol [138], the latter facilitating the budding process by providing proper membrane curvature [166]. The same mechanism of action has been described for lipid metabolism enzymes, neutral sphingomyelinase [153] and PLD2 [159] that act through generating ceramide and phosphatidic acid in the MVB membrane, thereby inducing negative curvature of the MVB and favouring intraluminal budding of the limiting bilayer.

2.4.1.3 EV secretion mechanisms

While most of the MVBs are destined to degradation through fusion with the lysosomes, some are directed to the cell membrane to release their content to the extracellular space. Their release is believed to be a regulated process requiring specific stimuli. For example, it has been proposed that similarly to lysosomal secretion, the intracellular calcium levels could play a role in plasma membrane fusion leading to exosome secretion from certain cell types [167]. However, the specific fusion machinery remains undefined. Additionally, several proteins are suggested to be involved in EV secretion (reviewed in [168]) with small GTP binding proteins of the Rab family (e.g. RAB11 [169], RAB27 A/B [170] and RAB 35 [171]) being important players in vesicle budding, mobility or tethering to the cell membrane. MVB fusion with the plasma membrane is potentially also aided by SNARE proteins [162], however the precise mechanisms of action has not yet been verified.

Accumulating evidence also suggests the presence of MVB subclasses with potential differences in their composition and fate [120,172–174]. Certain lipid content has been seen as a determinant for MVB subpopulation trafficking. For example, in case of B lymphocytes, cholesterol-rich MVBs appear to preferentially be directed towards the plasma membrane for subsequent fusion and exosomal release [175]. In contrary, lysobisphosphatidic acid (LBPA) a cone-shaped phospholipid which is generally absent on exosomes [138], resides in morphologically distinct MVB subpopulations destined for lysosomal degradation [176]. Exosomes with different molecular characteristics have also been detected at the basal and apical membranes of epithelial cells [177]. This type of intracellular trafficking and secretion is potentially modulated by V0-ATPase activity [178], yet the precise molecular mechanism of MVB polarized sorting remains to be established.

Thus, the secretion of specific MVBs is potentially dependent of several proteins as well as lipids. The existence of different MVB subclasses adds an additional layer of complexity to exosome biogenesis as these by themselves can contain an intermixed population of ILVs.

2.4.2 Plasma membrane derived EVs

In contrast to the endolysosomal pathway, subtypes of EVs (apoptotic bodies and MVs) are shed directly from the cell membrane. Though the generation of apoptotic bodies via plasma membrane blebbing has been known for decades [179] (and will not be discussed further), the mechanisms of MV release have just started to be unravelled. As opposed to exosomes, MVs arise via direct budding of the plasma membrane through a process that is activated by external factors such as extracellular calcium levels [180] and hypoxia [181]. Following induction, the calcium-dependent enzymatic machineries rearrange the phospholipids of plasma membrane, driving the exposure of phosphatidylserine (PS) on the cell surface. The latter causes restructuring of the actin cytoskeleton, a physical bend in the membrane and subsequent budding of MVs [182]. Notably, it has been found that the MV biogenesis might still take place even if the lipid asymmetry of the membrane does not change [183], indicating to a potential role of other lipid domains, such as cholesterol lipid-rafts [184] in the process. Recent finding suggests that a change in membrane curvature could also be aided by proteins [185]. Namely, the local protein "crowding" has been verified to generate lateral pressure through protein-protein interactions, thereby contributing to membrane bending at the cell periphery.

A successful biogenesis also demands well-regulated cytoskeletal dynamics, often mediated via RHO GTPases [186,187]. In addition, several alternative budding mechanisms including proteins such as ARF6 [188]; ESCRT component TSG101 (tumour susceptibility gene 101) together with arrestin domain-containing protein 1 (ARRDC1) [189], myosin-1a [182] and hyaluronan [190] have been implicated in the shedding of MVs, illustrating the richness of different pathways depending on the cell type and potentially the catalytic purpose that the shed MVs serve.

In summary, based on the current knowledge, multiple pathways seem to be responsible for the generation of EVs directly budding from the plasma membrane as well as the ones originating from the endolysosomal pathway. Whether there is a coexistence of different mechanisms, dependence on the cell type or whether different pathways are responsible for the production of distinct MVB, exosome or MV subtypes, is still to be elucidated.

2.5 Composition and loading of EV cargo

EVs have been recognized as vehicles of intercellular communication containing a significant amount of proteins, nucleic acids and lipids. This material largely reflects the molecular profile of their source cells and can be transmitted long distances to directly alter the

functional state of the recipient. Hereafter, the protein and RNA composition of EVs will be discussed in more detail, as the respective biomolecules were of central importance in the constitutive papers.

2.5.1 Protein composition of EVs

Proteins are the major bioactive components of the vesicular cargo, providing the bulk mass to density of EVs. The protein-rich composition is often exploited for characterization purposes in order to verify the type and purity of EVs. Nevertheless, as no exclusive proteins for the verification of exosomes or MVs exist, the enrichment relies on the testing of a combination of surface and luminal markers.

Due to the endosomal origin, almost all exosomes, independently of the cell of origin contain proteins related to MVB biogenesis (e.g. ALIX, TSG101, CD63, CD81, CD9) as well as membrane transport and fusion proteins (e.g. Rab GTPases, flotillin) [191,192]. These are however not exclusive exosome markers and some of them (e.g. CD63, CD81, CD9) are regularly also observed in MVs [140]. Due to the cytoplasmic biogenesis of both vesicle types, the purity of EV preparations is usually estimated by the depletion of membrane-binding extracellular proteins (e.g. fibronectin) and cellular proteins originating from the ER, Golgi and mitochondrial membranes [100]. Nevertheless, nuclear markers, such as histones or ER-derived chaperons (e.g. heat shock proteins (HSPs)) are still frequently detected in EVs, potentially aiding the EV-binding to heparin sulphate proteoglycans (HSPGs) or reflecting a general physiological phenomenon of tumour-derived vesicles [119,193–196].

Regardless that the protein content in exosomes and MVs is highly similar, it has been noted that the protein patterns of exosomes are more likely to differ from their source cells than the components of MVs [140]. Yet, the cargo of MVs still does not seem to be a random sampling of cellular components as several proteins such as ARF6 [188] and Rab22 [181] are postulated as potential components of the selective recruitment process. The incorporation of exosomal protein cargo has been studied more extensively, mostly relating it to the action of ESCRT components. The ESCRT machinery is known to detect and sequester ubiquitinylated (Uq) proteins to the endosomal membrane [197]. Even though the majority of ILVs with Uq proteins should be directed towards lysosomal degradation [198] ~60% of exosomal proteins still possess the degradation signal [199,200], hence making it logical to believe that the ESCRT components would be responsible for their enrichment in exosomes. Nevertheless, it has been seen that to a certain extent ESCRT components are unselective to ubiquitin binding, and also interact with non-Uq proteins such as transferrin [201]. This strengthens later findings that the binding to whichever ESCRT component might be enough for ubiquitin-independent cargo sorting [202]. The system becomes even more complex, as also the ESCRT components themselves can be Uq. This, however does not seem to be a critical aspect for their function, as a protein with a single ubiquitin moiety is sufficient to direct its sorting into MVBs [163].

Evidence of the involvement of ESCRT machinery in shaping the vesicular proteome can also be obtained from RNA inteference screenings, where the knockdown of HRS, TSG101 and STAM1 (signal transducing adapter molecule 1) affect the targeting of certain proteins to MVBs [174]. This supports an earlier study where the depletion of key subunits from the four ESCRTs caused perturbation in the MVB biogenesis and also prevented sorting of the epidermal growth factor receptor (EGFR) [163]. The involvement of the machinery is further supported by the fact that its components are constantly detected in EV preparations [140,158,200]. Still, protein cargo sorting is rather an intermixed process as only certain ESCRT components are known to affect the loading of a confined pool of proteins, suggesting the existence of alternative, ESCRT-independent mechanisms. For example, sorting machineries such as tetraspanin-enriched domains have been proposed to modulate the vesicular proteome [203]. The latter mechanism being especially intriguing considering that tetraspanins interact with a variety of transmembrane and cytosolic signalling proteins [204–206] and are highly abundant in exosomal protein profiles.

Thus, it is evident that the ESCRT machinery is involved in loading of at least certain proteins into EVs whereas parallel cargo sorting pathways are likely to contribute. Still, the precise loading mechanisms and their extent in shaping the protein signature of EVs remains obscure and might depend on the type of cell and/or vesicle being produced.

2.5.2 Nucleic acid composition of EVs

In addition to proteins, EVs also contain various types of nucleic acid. Findings on DNA include the detection of double stranded- and mitochondrial DNA [142,207,208] as well as retrotransposable elements [209]. As the presence of encapsulated DNA is still under active debate, the nucleic acid content will hereafter focus on different types of RNA and their loading mechanisms into EVs.

EVs incorporate a variety of RNA species including mRNA, transfer RNA (tRNA), mitochondrial RNAs (mtRNAs), long non-coding RNA and different types of small RNAs (miRNA, small nuclear RNA, small nucleolar RNA) [210–212]. Additionally, vault RNA (VT-RNA) an Y RNA fragments and mRNA degradation products have been identified [212,213]. Many studies are also reporting a strong predominance of ribosomal RNA (rRNA) fragments [214–217], often denoted as impurities in EV preparations. Yet, considering the precise cleavage pattern and overrepresentation of certain transcript regions in EVs [216,218], biological importance might still exist.

Packaging of EV RNA is much less understood than the loading mechanisms of vesicular proteins. Early studies suggested that the incorporation is a purely passive event [219], relating to the co-localization of miRNAs, Trinucleotide repeat-containing gene 6A protein (TNRC6A, also known as GW182) and AGO proteins within the MVBs [220]. EV miRNA association with RNA-induced silencing complex (RISC) proteins has also been investigated later, revealing an active and finely regulated signalling cascade responsible for miRNA

sorting via AGO2 [221,222]. The evidence supporting an active cargo loading arouse from *in silico* analysis [223] defining mRNA "zipcodes" which were later verified as YB-1 (Y-box binding protein 1) and NSUN2 [tRNA (cytosine(34)-C(5))-methyltransferase] recognition sites for exosomal mRNA and miRNA enrichment [224,225]. The presence of a similar zipcode has been also proposed for MVs [226], though the sequence is longer (25 nucleotides) and contains a different core string. The vesicular packaging of miRNAs could also be driven by hnRNPA2B1 (heterogeneous nuclear ribonucleoprotein A2/B1), which binds to specific "EXOmotifs" (GGAG or CCCU) [227]. Furthermore, other factors such as non-templated nucleotides at the 3' end of the miRNA [219] and ceramide-dependent packaging of miRNA have been suggested [228,229]. Non-random miRNA loading mechanism is also supported by the poor correlation of vesicular and cellular miRNA profiles (for some cell lines), the overrepresentation of certain miRNA molecules in EVs and altered levels of specific miRNAs in the serum of diseased individuals (which cannot be explained by pure passive secretion) [217,230,231]. Yet, some reports rather support passive secretion, indicating that the endogenous levels of natural miRNA targets modulate their secretion into EVs [232] and implying that some of the miRNA-binding proteins (e.g. AGO2) are relatively depleted in EV preparations [126,220, Paper II]. Thus, the mechanisms of vesicular RNA loading are rather inconclusive and suggest that parallel mechanism possibly coexist.

It has been suggested that the loading of RNA into exosomes starts at the endosomal membrane, where the RNA resides due to the membrane affinity determined by the RNA hydrophobicity, nucleotide sequence and lipid structure/composition of the site [233–235]. Considering the relative depletion of AGO2 in exosome preparations together with the 13-fold excess of miRNAs as compared to AGO2 [236], the membrane binding of "excess" miRNA might be plausible. On the other hand, other RNA binding protein (e.g. hnRNPA2B1) could also drive the miRNAs to the lipid raft regions owing to their affinity to ceramide-rich regions [227]. In terms of hydrophobicity, several RNAs are known to possess modifications (e.g. tRNA isopentenylation [233] and miRNA methylation [237]) which could potentially increase their affinity to raft-like membrane regions. Both miRNAs as well as tRNA-derived fragments are highly enriched in EV preparations [212,217,238]. Whether the hydrophobicity of these RNA stretches is also responsible for their sorting into ILVs or if the loading is rather mediated by RNA-binding proteins such as hnRNPA2B1 or YB-1[225] has not been fully addressed.

How exactly the selection and loading of RNA into exosomes occurs, remains debatable. The versatility of the proposed mechanisms is implying that the loading could be cell-type, physiological condition and even RNA-dependent, all this making it plausible that the observed mechanisms may coexist. The wider availability of next generation sequencing techniques greatly facilitates nucleic acid research, thus improved understanding of the composition and molecular secretion mechanisms of extracellular RNAs into EVs it is well on its way.

2.6 EV uptake

In addition to verifying the mechanisms of macromolecule loading, increasing attention is paid on EV uptake mechanisms, being of high importance for the development of therapeutic interventions.

2.6.1 Methods to track EV uptake

The monitoring of EV uptake *in vivo* generally relies on the exploiting of lipophilic fluorescent dyes giving an overall distribution and relative quantification of cell-incorporated material [239]. This strategy is however prone to pitfalls including unspecific labelling of other membrane limited particles, lateral transfer of the dye between cells and extended half-life beyond that of EVs [240,241]. Single EVs, on the other hand are commonly tracked by surface engineering of EV-enriched markers with fluorescent proteins [147]. However, as the heterogeneity of EVs limits the tracking of all vesicle subpopulations by this methodology, general membrane labelling strategies using palmitoyl-fluorescent protein fusions have successfully been exploited [242]. The latter strategy labels the inner leaflet of the EV membrane, hence impact on EV binding and uptake is expected to be minimal. In *in vivo* settings, EV uptake has also been tracked with the Cre-loxP system [243]. Though this methodology demands the generation of donor- and recipient reporter cells, it represents a highly sensitive approach for tracking the functionality of EVs in physiological conditions.

2.6.2 Mechanisms and influence of surface molecules

EV uptake occur via multiple routes, including a direct fusion with the cellular membrane [244], as well as EV internalization via lipid raft-, clathrin- and calveolae-dependent endocytosis, micropinocytosis and phagocytosis [147,184,245,246]. The exact mechanism likely depends on the specific lipid/protein composition of the EVs and cell membranes, the local microenvironment (e.g. acidic *versus* basic) and potentially also on the EV subtype [247].

It has long been postulated that EVs, depending on the composition of their surface molecules, possess an inherent cellular homing ability. Implications for such a behaviour have been observed with unmodified dendritic cell (DC) EVs (Paper IV) as well as tumour-derived EVs [248,249], exposing an organotropic behaviour *in vivo*. Cell-pairing has also been noted *in vitro* (personal communication with Dr. Nicole Meisner-Kober and Dr. Wolf Heusermann), nonetheless at times contradicting with the evidence from *in vivo* screenings [250] and thereby indicating to the need of further investigation.

Various complexes are known to use HSPGs to gain entry to the cell, EVs not being an exception [251]. Treating the cells with heparin sulfate (HS) mimetics (e.g. heparin, dextran sulfate) has been seen to block EV transfer to recipient cells, whereas the presence of HSPGs on EVs has no apparent effect on their internalization [251–254]. Owing to the polyanionic nature of HS mimetics, the inhibition of the cell entry is potentially an intermixed process and could also include binding to scavenger receptors (SRs). For example, SR-mediated effect has been observed for high density lipoprotein (HDL) nanoparticles [250], which in contrast

to the competitive binding mechanism of HS, deplete cholesterol from the cell membrane, induce SR clustering and thereby inhibit EV uptake. In addition, many studies have reported the importance of C-type lectins on EV uptake [255,256]. Importantly, in *in vivo* context, healthy pancreatic tissue near pancreatic ductal adenocarcinoma (PDAC) is known to release a specific type of lectin, which binds to tumour-derived EVs (tEV), interferes with their uptake *in vivo* and inhibits metabolic changes in cancer cells [250]. Moreover, protein and integrin signatures are known to be important for EV uptake [248,257,258], illustrating the variety of molecules potentially affecting internalisation of EVs to recipient cells.

2.6.3 Factors influencing EV uptake *in vivo*

Despite a lack of understanding on the secretion and uptake mechanisms of physiologically produced EVs, it is evident that these processes are finely regulated to evade systematic clearance of naturally produced EVs. Yet, due to the complexity of monitoring EV secretion and uptake in physiological settings (e.g. using xenografts), a great majority of *in vivo* studies are based on exogenously produced vesicles. The curiosity in vesicle *in vivo* uptake mechanisms and surface molecules has largely been driven by the therapeutic potential of EVs, as by modulating EV trafficking, one could enhance targeted biomolecule delivery or block the transfer of EVs carrying malignant features.

Intravenously administered EVs have an *in vivo* half-life between 2 minutes to 24 hours [259–263]. In the body, the EVs are constantly patrolled by a network of cells constituting the MPS, the system responsible for detecting and clearing foreign material. The scope of this clearing machinery can be illustrated by the common biodistribution pattern of exogenously administered EVs, which follow a signatory MPS clearance with EV accumulation in spleen, liver and lungs [259,262,264]. The extent by which tissue resident macrophages clear systemically injected EVs is impressive, as macrophage-depleted mice are able to clear only < 2% of EVs as compared to control animals [265]. Similarly to liposome clearance [266,267], MPS elimination pattern can potentially be attributed to SR activity [268], representing one of the mediators of EV uptake, as discussed earlier. The blockade of SRs with dextran sulfate has been shown to decrease liver uptake, significantly increase the amount of circulating EVs in plasma and promote vesicle accumulation in breast cancer tumours [269]. The SRs on the surface of macrophages may also become activated by the exposed PS on the outer leaflet of the EV membrane [270–272]. Owing to the membrane curvature, the enrichment of PS might only be apparent and result from the high membrane curvature of the vesicles, rendering two thirds of the lipids to be placed in the outer leaflet [273]. Though contradicting thoughts about the precise exposure time, mechanism and physiological purpose of PS in the EV membrane remain (reviewed in [273]), PS is known to play a key role in phagocytosis and could thereby also affect the circulation time of EVs in the body.

Another major factor affecting the uptake and biodistribution of exogenous EVs is vesicle aggregation. This can arise from high *g* forces used in the ultracentrifugation-based protocols or result from EV enrichment methods affecting the natural surface signature of the vesicles

(e.g. protein corona [274]), leading to decreased molecular shielding and aggregation of EVs [114]. The aggregation is known to modulate the EV uptake routes, cause notable differences in the biodistribution and from a therapeutic point of view, could decrease the efficacy of the preparation [114]. It has also been suggested that EVs can aggregate in the biological fluids due to calcium-mediated cross-linking of PS, similarly to observations with liposomes [275], to my present knowledge, studies exploring this possibility on EVs have thus far not been conducted. To (re)shield the EV surface, polymers such as PEG can be exploited [275]. The hydrophilicity and biocompatibility of PEG make it an excellent tool to simultaneously decrease vesicular aggregation and increases EV circulation half-life by shielding them from interactions with plasma proteins. Nevertheless, due to the physical blocking of protein-protein interactions, PEG might dampen the EV-mediated response, unless surface-extended targeting moieties are employed [275].

The body is a rather hostile environment for exogenously administered EVs with a multitude of clearance mechanisms preventing the vesicles from reaching its target. Only through obtaining a detailed knowledge of the physiological EV uptake and clearance mechanisms *in vivo*, one would be able to hijack the inherent pathways and aid the progress of EV-mediated therapies.

2.7 Biological role of EVs

EVs have been found in all analysed biological fluids and in the conditioned medium of cultured mammalian cells [276–278]. Nonetheless, the universal cellular secretion of EVs extends far beyond eukaryotic multicellular organisms, also covering simple organisms such as bacteria and archaea [279,280] and thereby suggesting an evolutionarily conserved mechanism of cellular communication [281].

2.7.1 EVs from prokaryotic organisms

Though this review concentrated on EVs derived from mammalian (mostly human) cells, it is important to note that the production of EVs already existed in prokaryotic organisms.

While EV secretion by archaea has been studied relatively little, these organisms are known to secrete membrane vesicles of 50-230 nm in size, originating from the cytoplasmic membrane [282,283]. The precise role of archaeal EVs has not been fully elucidated, nevertheless EVs from monoderm archaea are known to convey antimicrobial activity by carrying proteins which inhibit the growth of related archaea species [279,282]. Moreover, the molecular content of EVs from the archaea family Thermococcales also includes DNA [284]. Considering the extreme conditions these hyperthermophilic archaea live in, the vesicles provide an excellent protection for the nucleic acid against thermodenaturation and furthermore, similarly to eukaryotes, mediate its transfer between different archaea species [283]. Interestingly, also archaea possessing a double membrane secrete EVs and are often in

close association to other organisms, making it possible that the secreted vesicles deliver essential components (e.g. lipids) between the two symbionts [285].

In bacteria, EVs act as potent virulence factors [284]. By containing DNA, toxins, immunomodulatory compounds and adhesins, bacterial outer membrane vesicles (OMVs) are known to contribute to the colonization and successful transmittance of virulence factors to the host organism. Notably, the production of EVs by bacteria is not only limited to infection, but also carries on during and after the transition to bacterial biofilms [286]. There, the vesicles contribute to the biofilm nucleation, nutrient acquisition, defence and bacterial communication, thereby serving as potential targets to therapeutic interventions. EVs containing DNA, RNA and proteins have also been isolated from bacteria in the natural environment, such as the marine ecosystem [287], where the vesicles act as an organic carbon source to other marine bacteria, serve as external protein receptors to tackle phage infection or again, plausibly constitute vectors for horizontal gene transfer. Hence, most the basic biological mechanisms attributed to vesicles date back further beyond the arrival of eukaryotes, which nonetheless have attained a spotlight in EV research and will be discussed hereafter.

2.7.2 EVs from eukaryotic organisms

The initial lack of interest in EVs was dependent on the knowledge which considered them as means of disposing unwanted material from cells. 40 years after their initial discovery, the vesicle field was rediscovered thanks to the work of Raposo *et al.* [18] describing the communication between B cell-derived EVs and T cells and indicating to the functional role of EVs in cell-to-cell communication. From then onwards EVs have been purified from different eukaryotic organisms including plants (e.g. carrots, ginger, lemon, grapes, grapefruit, watermelon and olive pollen grains [288–293]), fungi [288] and animals [294–296]. In plants, EVs have been shown to mediate interspecies communication for example by modulating the expression of genes such as anti-inflammatory cytokines that are crucial for maintaining intestinal homeostasis [289,290] or transferring cross-kingdom dietary miRNAs [291]. In fungi, similarly to bacteria, the EVs are known to mostly transport virulence associated components [297–299], enhancing pathogenesis even across the blood-brain-barrier [291]. Most of the vesicular studies concentrate on EVs from animals, especially humans and common model organisms such as mice with the bulk number of studies being performed in cell-cultures [112]. Due to the variety of EV origins, the discussion of their biological roles is unfortunately out of the scope of this review and will instead give a brief overview of the role of EVs derived from mammalian, mostly human and mouse cells.

EVs have been isolated from a range of body fluids including, among others, blood components, saliva, amniotic fluid, breast milk and urine [276,278,300]. Their role in the body includes a range of physiological processes such as fertilization, development, tissue regeneration, stem cell maintenance and immunomodulation which are all achieved by protein interactions with cell surface receptors and delivery of bioactive RNA-, protein- and lipid cargo (reviewed in [301]). These features are also exploited by EVs derived from

diseased cells, such as tEVs which use the potency of vesicular signalling to promote immune escape, stimulate growth and support disease spreading, thereby serving as good candidates for therapeutic interventions [302].

EVs play and important role already at the early stages of development. At fertilization, EVs are known to promote sperm-egg fusion [303] and thereafter modulate migration and implantation of the early embryo [304]. Subsequently, thanks to the encapsulated morphogens (e.g. Wingless (Wnt) and Hedgehog) EVs play an active role in establishing the body plane and tissue organization and thereafter support the physiological processes throughout the lifecycle of the organism [162,305].

One of the most widely explored fields of EV communication relates to various functions of the immune system. For example, the role of EVs has been implicated in the induction of T-cell activation and differentiation modulation towards T helper 1 phenotype, thereby enhancing *in vivo* immunogenicity [306–308]. EVs also aid in maintaining an immune-privileged site at pregnancy by suppressing T-cell signalling components [309], mediate immune activation in response to allogeneic organs and tissues [310] and participate as pro-inflammatory mediators in the pathophysiology of arthritis [311], preeclampsia [312] and sepsis [313]. Tumour cells are known to secrete anti-inflammatory tEVs by carrying ligands which induce T-cell apoptosis or suppress their cytotoxicity and thereby facilitate the generation of tumour-promoting immunity [314]. Interestingly, the treatment with anticancer drugs induces the secretion of tEVs carrying HSPs, which in contrast are able to generate CD8+ T-cell and/or natural killer cell dependent antitumor effect [315,316]. Apart from immunomodulation, tEVs play an important role in the generation of an extended tumour microenvironment by stimulating angiogenesis, promoting cell migration and inducing vascular leakiness [317,318]. All these processes modulate the local physiological conditions for tumour thriving and furthermore, aid its dissemination, again with the help of tEVs that pre-condition distant sites for metastatic lesions [41,319,320].

The majority of the aforementioned biological roles of EVs depend on the interactions between the vesicular and cellular macromolecules, mostly proteins. Protein transfer in physiological settings has been implicated in a recent study investigating the tissue crosstalk of EVs during exercise, showing that physical exercise provokes the secretion of EVs with altered protein cargo which is mostly directed to the liver [321]. Notably, these proteins also included several novel candidate myokines, providing evidence of a new mechanism of myokine secretion. Owing to the high efficiency of this macromolecular communication system, vesicular trafficking also affects the course of pathogenesis for many diseases. For example, EVs have been implicated in the progression of several neuronal conditions like Alzheimer's disease and Parkinson's disease by spreading pathogenic proteins α-synuclein [322] and amyloid-β [323,324].

The list of functions attributed to EVs also includes the encapsulation and delivery of nucleic acids. As the majority of vesicular RNA content does not exceed 200 nucleotides [143,144], many of the physiological roles are related to the transfer effects of short RNAs, especially

miRNAs. For instance, the majority of miRNA in blood is known to originate from adipose cells and circulates in association with EVs [325]. Some of these miRNAs have been shown to play an important role in metabolism regulation by improving glucose tolerance via gene expression modulation in liver via fibroblast growth factor 21 (Fgf21) [325]. Furthermore, EVs derived from adipose tissue macrophages are able to transfer insulin resistant traits from obese subjects to lean mice by carrying miR-155 which contributes to the phenotypic changes [326]. According to recent studies, EV miRNAs are also means of controlling the ageing of stem cells, directing the balance of cellular differentiation, inducing cellular repair programs and repressing apoptotic genes [327–331]. At the immune synapse, T cell-derived EVs release vesicles that contain miRNA and are able to modulate gene expression in antigen presenting cells (APCs) [303]. The exchange of miRNA cargo has also been noted between DCs and DC EVs [303] as well as regulatory and helper T cells [303]. MiRNAs in tEVs are known to promote tumour progression by modulating metabolism and immune response, promoting angiogenesis, inducing metastasis and conferring resistance to therapy [332–338]. The role of many other EV RNA species such as tRNA fragments, Y RNAs, circular RNAs and long noncoding RNAs (lncRNAs) has been less studied and their physiological role is yet to be established. Nevertheless, EV associated lncRNAs have been implicated in the enhancement of cell viability, tumour growth and progression [339,340], tRNA halves in maintaining stem cell potency to differentiation [341] and Y RNA fragments in cardioprotection via modulation of interleukin-10 (IL-10) expression [213].

The fact that all cells tested from the three domains of life produce EVs indicates to their indispensable role in supporting physiological processes. Different cell types are continuously exchanging EVs over short and long distances *in vivo*, making it a rich source of information for the surveillance of health and disease and serving as basis for therapeutic interventions.

2.8 Therapeutic potential of EVs

Owing to the surrounding lipid bilayer, the content of EVs is well protected from nuclease and protease degradation, enabling long distant transport of macromolecules to exert remote extracellular communication. Though this could also be achieved by other delivery vehicles, such as liposomes, EVs hold features that stay beyond the reach of synthetic carrier systems, such as native biological components serving as a rich source of intrinsic therapeutic and biomarker-based potential. Potentially more importantly, they have an intrinsic ability to cross several biological barriers that their synthetic counterparts are unable to do.

The first reports describing the potential of EVs in ameliorating diseased conditions arose from studies employing the conditioned medium (CM) of hypoxic MSCs to relieve acute myocardial infarction [342,343]. Thereafter, many studies have employed the inherent therapeutic potential of MSC EVs to treat a variety of conditions including therapy-refractory graft-versus host disease (GvHD), radiation damage and kidney injury [92,128,344–347]. The healing potential of MSC EVs has been postulated emanate from the delivery of

proteins/RNA to promote angiogenesis and suppress apoptosis, generation of energy needed for cell survival and immunomodulatory properties [253,347–349]. The administration of stem cell-derived EVs has been confirmed safe in humans as well as in all tested animal models [128,344,350–352], yet clinical results in humans have only been conducted on two instances [128,350]. Currently, allogenic unmodified MSC EVs from cord blood are undergoing Phase II/III clinical trial of Type-1 diabetes (NCT02138331), but the results of that trial are yet not published. Considering the successful employment of MSC-derived EVs in experimental animal models, more trials are expected in the near future.

Another vesicle source often used in therapeutic setting is DCs, which are commonly used for the generation of EV-based cancer vaccines. For example, tumour-associated antigen pulsed DC EVs have been used in cancer immunotherapy against non-small-cell lung cancer [353,354], colorectal cancer [355] as well as metastatic melanoma [356], all these clinical studies implicating to a safe and well-tolerated T-cell mediated antitumor response. In other settings, vesicle-based vaccines have been derived from bacteria and yeast cells [357,358]. Wild type OMV-based vaccines from bacteria were developed already more than 20 years ago [359] and are known to exhibit a remarkable immunomodulatory potential. Though no fungal vaccines have yet reached to the clinic, currently four licensed OMV vaccines from *Neisseria meningitidis* serogroup B are available [357,359,360].

In addition to vaccines, EVs have gained increasing interest as vehicles for nucleic acid delivery. Due to the inefficient loading of larger stretches of RNA [361], the majority of studies rather exploit the therapeutic potential of short RNA molecules. For instance, MSC and fibroblast EVs loaded with short RNAs against oncogenic KRAS have been reported to efficiently target pancreatic cancer in multiple mouse models and significantly increase the overall survival of the animals [362,363]. The potential of EV miRNAs also arises from studies showing that EV-encapsulated miRNAs are able to enhance the therapeutic effect of MSC EVs in ameliorating liver fibrosis [364], inhibiting myeloma-related angiogenesis [365] and supporting liver protection in experimental autoimmune hepatitis [366]. Similar therapeutic effect has been achieved by using EVs loaded with mRNA/protein. An interesting solution to antitumor therapy was reported by Mizrak *et al.* [367] where MVs were engineered to carry a suicide gene mRNA/protein which was able to convert a nontoxic prodrug 5-fluorocytosine to an anticancer agent 5-fluorouracil and thereby efficiently induce tumour regression upon systemic treatment with the prodrug.

Irrespective of the efficiency of the bioactive RNA molecule, it is of utmost importance that the majority of the cargo reaches to the site of interest. To escape the highly efficient EV clearance mechanisms *in vivo*, EV surface has been modified with PEG molecules or ligands such as CD47, which acts as a "don't eat me" signal for phagocytic cells and enhances EV circulation half-life in blood [362,368]. The therapeutic effect of EV-loaded cargo can further be improved by modifying the repertoire of surface molecules. Though the employment of targeting moieties is often not necessary to gain a biological effect, surface engineering strategies greatly reduce off-target events and enhance uptake specificity. Surface modification of EVs has for example been used to target EGFR expressing breast cancer cells

with let-7a miRNA [263] and to direct vesicular siRNA delivery to the brain by using rabies virus glycoprotein (RVG)-targeted EVs [79]. Avoiding off-target effects is especially important for some synthetic drugs, such as chemotherapeutic agents. By employing an arginine-glycine-asparagine targeting peptide, doxorubicine-loaded EVs have been reported to selectively accumulate in breast cancer xenografts and reduce tumour growth [369]. At times, chemotherapeutics (doxorubicin, curcumin or paclitacel) have also been transferred via untargeted EVs resulting in successful inhibition of cancer growth and amelioration of septic shock in mouse models [369–373].

In addition to targeted therapeutics, EVs could be employed as diagnostic (and prognostic) biomarkers. The largest medical area possibly benefiting from EV-based non-invasive disease monitoring relates to cancerous conditions. Altered expression patterns of several EV miRNAs are known to contribute to the pathogenesis and metastasis of a range of tumours [334,374–381] as well as to neurological disorders [338,382]. Specific protein composition of EVs has also been associated with the transfer of metastatic potential to non-cancerous cells and plausible serves as a determinant of metastatic organotropism [41,248,319]. As many cancers are not easily accessible for histological analysis, the sampling of EVs and profiling of their macromolecule content allow non-invasive diagnostics for primary tumours, metastasis, cancer relapse as well as response to therapy [383,384]. Currently, implications for EV miRNA-based prognostics have only been reported for prostate- and ovarian cancer [375,377].

The natural therapeutic properties together with infinite opportunities of EV modifications have paved the way for an explosive interest in EVs as biomarkers, gene expression regulators and macromolecular drug delivery vehicles. The progression from bench to bedside is somewhat hampered due to the lack of standardization in isolation and characterization methods as well as insufficient knowledge about their *in vivo* biodistribution, stability and clearance. Additionally, sufficient expertise on their biological role is still needed before translation into clinics can be achieved.

2.9 EVs *versus* synthetic lipid-based delivery systems

Lipid-based delivery systems have been employed for drug delivery already for decades whereas the employment of EV-based therapeutics in clinical setting is still in its infancy [385]. Both of these delivery systems share a range of common features in their composition, uptake and biodistribution as well as exploit common methodologies for isolation and characterization. Despite the high resemblance to one another, these natural and synthetic vesicles have a range of dissimilarities which could be exploited to further improve both systems.

Lipid-based formulations are the most studied for drug delivery with numerous approved products mostly to deliver chemotherapeutics [386,387]. The number of clinical studies performed with cell-derived EVs is much more sparse and clinically approved EV-

therapeutics do not yet exist [351,388]. One of the major hurdles in translation of EVs to clinical use is the development of well-controlled, standardized, high-yield production, which is achievable for synthetic vehicles, such as liposomes [389], but considerably more complex in case of biological systems [389]. Apart from that, EV-based therapeutics are often difficult to define in terms of the mode of action underlying the suggested effect. This results from the highly complex macromolecule composition of EVs, serving as a rich source of therapeutic potential, yet making it virtually impossible to define and constantly reproduce the set of active components. Such hurdles are largely unfamiliar to highly defined synthetic carriers, which however could greatly benefit from molecular characteristics of EVs to improve their performance.

Despite the synthetic or natural origin of the aforementioned carriers, both delivery systems can be considered as nanoparticles allowing many of the technologies that have been in use for lipid-based delivery systems (liposomes) to also be exploited in EV research. This applies for example to the generation of artificial EVs via extrusion through filters or by using microfluidic system [370,390]; purification methods such as ultrafiltration, UC and SEC [114] as well as to analytical methods measuring the size, surface charge and concentration of the nanoparticles [391]. Similar lines in the two carrier systems can also be seen in the range and strategies of cargo loading. Both EVs and liposomes are lipid bilayered vesicles with an aqueous core, serving as a suitable environment for the encapsulation of hydrophilic small molecules as well as macromolecules, as already discussed. The encapsulation of hydrophobic molecules is somewhat more difficult for EVs [392], whereas the preparation procedure of synthetic liposomes seems tailored for such purpose, allowing integration of hydrophobic cargo into the bilayer during assembly [389]. In terms of cargo loading strategies, some loading (e.g. endogenous overexpression and microfluidic mixing) are characteristic to natural or synthetic nature of the nanoparticles and can therefore not be cross-applied. On the other hand, simple co-incubation with the cargo of interest can be used for both EVs and liposomes [371,393,394].

In addition to various similarities in the range and strategies in cargo loading, EVs and liposomes are both amenable to modifications that allow the cargo to reach its target in a more specific manner. Whereas EVs already hold a wide selection of surface molecules, potentially acting as combinatorial targeting moieties, liposomal surface ligands need to be selectively tailored for the purpose. In return, liposomal formulation procedure allows controlling the affinity, avidity as well as receptor density on the surface [395]. This makes targeted liposomal delivery more predictable than the transfer via EV-mediated systems, which suffer from heterogeneity and undefined representation of the ligands [396]. It has been observed that in head-to-head studies liposomes often succumb to EV-mediated delivery [264,394,397]. Yet, many studies seem to lack fair grounds for the presented comparisons as the liposomal formulations are often inadequately chosen or poorly described [396]. Hence, the potential superiority of EVs over synthetic carriers perhaps cannot be ascertained as the benefits of both systems are highly dependent on the macromolecule and disease context.

Despite apparent differences in the abundance, complexity and pattern of surface molecules, these ligands affect the *in vivo* fate of both liposomes as well as EVs. For example, one of the lipid components of EVs, PS is commonly employed also in liposomal formulations and might partially determine the circulation half-life of both nanoparticles. Physiologically, PS is known to trigger phagocytosis of apoptotic cells, and as illustrated by the biodistribution pattern of both EVs and liposomes, also acts as a mediator of MPS-driven nanoparticle clearance, rendering the majority of the material to accumulate in liver and spleen [259,398–400]. This accumulation pattern could in part also be attributed to the similar size range of EVs and liposomes, where smaller particles (up to 100 nm) accumulate in the liver and larger ones (> 200 nm) are removed from the circulation by splenic filtration [401,402]. Nevertheless, lipid composition and size represent just a part of the biophysical properties that liposomes and EVs hold, as also surface charge, -hydrophobicity and protein composition are major effectors of successful delivery.

In order to evade clearance by biological surveillance mechanisms, the surface of liposomes is often modified by PEGylation [403]. Coating with polymers has successfully also been applied for EVs and provided an effective means of prolonging circulation time *in vivo* [368]. EVs are postulated to also escape clearance owing to the highly heterogeneous protein decoration, simultaneously promoting extended circulation time and acting as ligands to enhance specific uptake by target cells [399]. Liposomes, in contrast, generally do not have surface proteins or possess only one specific targeting ligand, limiting the variety of specific ligand-receptor interactions and often forcing them to solely rely of surface PEGylation to avoid removal from circulation.

The benefit of using biological systems as delivery vehicles includes their high versatility in the diversity and characteristics of natural molecules. This heterogeneity serves as an inherent source of potential therapeutics, yet makes it often hard to define and reproducibly generate the active component in the future. Liposomes represent a more controllable system in terms of their size, composition and biophysical properties, yet suffer from the lack of innate combinatory targeting potential and higher toxicity. An increased understanding of the components and gateways that EVs use for signalling and cell entry would hence open new avenues for the delivery of bioactive cargo which could be hijacked by lipid-based delivery systems offering a defined environment for the biologics.

AIMS

Lipid-based delivery vectors are one of the most widely exploited nanoparticles for the encapsulation of nucleic acids. This thesis firstly investigates the nucleic acid delivery potential of synthetic cationic lipid-based nanoparticles and thereafter concentrates on the composition and content dynamics of their natural counterparts, EVs, in order to shed light on the potential of lipid-based entities for therapeutic nucleic acid delivery. The individual objectives of the constituent papers were set to serve the wider aim of this thesis and are the following:

3 Paper I

- To evaluate the extent by which cryo-manipulation affects the nucleic acid delivery efficiency of cationic lipid-based reagents.
- To shed light on the mechanisms contributing to the elevated potency of the cryo-manipulated formulations.
- To understand the applicability of freeze-thawing on the delivery potential of a wider range of commercial formulations.

4 Paper II

- To investigate the cell line dependant differences in the small RNA and protein composition of EVs.
- To examine the interplay of vesicular RNA and protein content with a special focus on RNA/miRNA binding proteins and the respective RNA species.

5 Paper III

- To investigate the overall small RNA content in EVs and non-vesicular secretome.
- To verify the extracellular profile and quantity of secretory miRNAs at their basal level as well as upon genetic overexpression.
- To describe the differential sorting patterns of secretory miRNAs and their importance to the biological activity of EVs.

6 Paper IV

- To investigate how the dose and route of administration affects the biodistribution of EVs *in vivo*.
- To investigate cell source dependent tissue distribution and inherent homing capacity of EVs.
- To explore the effect of a targeting moiety on the general EV biodistribution and local accumulation to the tissues of interest.

METHODOLOGY

7 Methodological considerations

Detailed descriptions of the employed methodologies can be found in the constitutive papers. The following chapters aim to give a concise overview of the most important materials and methodologies used.

7.1 Cell sources

HEK293T (human embryonic kidney cells), Neuro2a (mouse neuroblastoma cells), human spinal muscular atrophy fibroblasts (GM03813), HepG2 (human hepatocellular carcinoma cells), C2C12 (immortalized mouse myoblasts) and hTERT-MSCs (human telomerase reverse transcriptase immortalized bone marrow mesenchymal stem cells) [404] were included in Paper I to evaluate the nucleic acid delivery efficiency of synthetic lipid-based transfection reagents. The small RNA and protein content of EVs (Paper II) was investigated in HEK293T, RD4 (human skeletal muscle cells), Neuro2a, C17.2 (immortalized mouse neural progenitor cells) and C2C12 cell lines. Paper III focused on human derived cell sources and exploited the fractionation of the extracellular material derived from HEK293T cells and hTERT-MSCs. Paper IV employed the conditioned medium of murine bone marrow derived DCs (strain C57BL/6J), hTERT-MSCs, rat oligodendrocytes (OLN-93), C2C12 cells and mouse melanoma cells (B16F10) to evaluate the *in vivo* biodistribution of EVs in C57BL/6J mice. The culturing conditions of the aforementioned cell types are outlined in the respective papers.

7.2 EV enrichment techniques

Paper II and IV include ultracentrifugation as the chosen method for EV enrichment. Briefly, the cell culture CM was first subjected to differential centrifugation steps whereby the clearance of floating cells and cell debris was achieved at 300 × g and 2000 × g, respectively. Thereafter, in order to enrich for smaller vesicles the supernatant was cleared through 0.22 µm filter followed by pelleting of the EVs at ~120 000 × g and a subsequent wash spin of the phosphate buffered saline (PBS) reconstituted pellet at 120 000 × g. The final EV pellet was brought to a desired volume with PBS. In order to allow improved fractionation of the extracellular secretome in Paper III, we employed size exclusion liquid chromatography methodology[114]. The workflow included the aforementioned differential centrifugation steps as well as a 0.22 µm filtration step, resulting in cell culture media largely deprived of larger particles. The cleared CM was then ultrafiltrated by using 100 kDa MWCO filters (Amicon, Merck Millipore) at 3500 × g. The resulting retentate was loaded onto HiPrep 16/60 Sephacryl S-400 HR column (GE Healthcare, PA, USA) by employing the ÄKTA pure/prime chromatography system (GE Healthcare, PA, USA) equipped with a 280 nm UV

detector. The EV and non-vesicular samples were pooled based on the resulting absorbance profile and concentrated using the 10 kDa MWCO Amicon Ultra spin filters (Merck Millipore). In order to cover a wide range of extra-vesicular material, the non-vesicular sample also included the flow through from the 100 kDa ultrafiltration step.

7.3 Nanoparticle characterization

7.3.1 Nanoparticle Tracking Analysis

To measure the size distribution and concentration of particles, all constitutive papers employed Nanoparticle Tracking Analysis (NTA) on the NanoSight NS500 instrument (Malvern Ltd, UK) equipped with a 488 nm laser and a 500 nm long pass filter. The specific settings in terms of camera gain, shutter setting and detection threshold are described in the individual papers. Generally, five 30-60 seconds long videos per sample were recorded and analysed with NTA 2.3 analytical software (Malvern Ltd, UK). For the quantitation of fluorescent particles, the sample was under a constant flow in order to decrease the bleaching of the fluorescent signal.

7.3.2 Western blot

The EVs in Paper II, III and IV were evaluated for the enrichment of EV markers with WB. Briefly, an equal number of particles (5E9-1E10) were mixed with the sample buffer containing 0.5M dithiotreitol (DTT), 0.4 M sodium carbonate (Na_2CO_3), 8% sodium dodecyl sulfate (SDS), 10% glycerol and thereafter heated at 65ºC for 5min. The cell samples were scraped from 2D cell culture plates, counted for viability with Trypan Blue Exclusion test (Thermo Fisher Scientific, MA, USA), pelleted and lysed with an appropriate volume of radioimmunoprecipitation assay (RIPA) buffer, kept on ice and vortexed every 5 min for half an hour. Subsequently, the samples were spun at 12 000 × g for 12 minutes at +4ºC; the supernatant was collected, mixed with the sample buffer and heated as described above. The samples were run on NuPAGE Novex 4-12% Bis-Tris Gel (Invitrogen, Life Technologies) at 120V and transferred to an iBlot nitrocellulose membrane with the iBlot system (both Invitrogen, Life Technologies). The membranes were blocked with Odyssey blocking buffer for 60 minutes at room temperature with gentle shaking. Thereafter, the membranes were probed with primary antibody solutions described in the respective papers, followed by IRDye 800CW and 680LT secondary antibody solutions (LI-COR Biosciences, NE, USA). Between the aforementioned steps, the membranes were washed every 5 min for half an hour with washing buffer (1 × PBS with 0.1% Tween 20; Sigma Aldrich, Saint-Louis, MO, USA). Washing steps including a final rinsing of the membrane with 1 × PBS were also performed before imaging. The membranes were visualized on the Odyssey infrared imaging system and further analysed by using Image Studio Lite Version 5.2 (both from LI-COR Biosciences, NE, USA).

7.3.3 Total Internal Reflection Fluorescence Microscopy

The physical characteristics of lipoplexes in Paper I were evaluated with Total Internal Reflection Fluorescence (TIRF) microscopy on a Zeiss Laser TIRF 3 system (Carl Zeiss AG, Germany). The deposition of Alexa-568-labeled duplex RNA complexes with freeze-thawed or non-frozen LF2000 (Thermo Fisher Scientific, MA, USA) on P35G-1.5-14-C glass inserts (MatTek Ashland, MA, USA) were recorded at 37 °C in preheated Opti-MEM Reduced Serum medium (Thermo Fisher Scientific, MA, USA). The time dependent complex deposition on the glass surface was expressed as increase in the relative fluorescence over the basal background.

7.3.4 Transmission Electron Microscopy

TEM was utilized to characterize the lipoplexes in Paper I as well as EV morphology in Papers II, III and IV. In all cases, 2% uranyl acetate solution (Sigma-Aldrich, Saint-Louis, MO, USA) was used to stain the material spotted onto glow discharged electron microscopy grids. In some experiments of Paper I, a biotinylated nucleic acid (Label IT Nucleic Acid Labelling Kit, Biotin, Mirus Bio LLC) and 10 nm colloidal gold labelled neutravidin were used to visualize the formed complexes for their identification. The imaging was performed by using a FEI Tecnai G2 Spirit BioTWIN microscope (FEI, OR, USA) run at 120 kV (Paper I), with a FEI Tecnai 10 TEM (FEI, OR, USA) at an accelerating voltage of 100 kV (Paper II, III) or JEOL 1010 TEM (JEOL, Peabody, MA, USA) (Paper IV).

7.4 Methods to evaluate nucleic acid loading and delivery efficiency

7.4.1 Luciferase assay

Transfection efficiencies of different synthetic delivery vectors in Paper I were assessed by using the Luciferase Assay System (Promega Corporation, WI, USA) and luminescence of firefly luciferase was measured with GloMax-96 Microplate Luminometer (Promega Corporation, WI, USA). When applicable, the luciferase activity was further normalized to the amount of total protein in each sample, as measured by a Protein Assay (Bio-Rad Laboratories, Hercules, CA).

7.4.2 RT-qPCR

Paper I and III included RT-qPCR analysis to either determine the potency of nucleic acid delivery vehicles or quantify the miRNA content of the secretory fractions. In Paper I, the recipient cells (HeLaLuc705[405]) were treated with splice-correcting minicircle plasmids or oligonucleotides to restore the correct splicing and generate a functional firefly luciferase in the aforementioned reporter cells. In Paper III, RT-qPCR was employed to either monitor the mature miRNA level of overexpressed let-7a and let-7b in the different fractions of the HEK293T secretome or to quantify the basal expression of a selected set of miRNAs in HEK293T and hTERT-MSC EVs and non-vesicular fraction. In both papers, total RNA was

extracted following the standard phenol-chloroform extraction protocol by using either Trizol or Trizol LS solutions (both from Thermo Fisher Scientific, MA, USA) followed by cDNA synthesis using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, CA, USA). U7asLuc705 small nuclear RNA and small nucleolar RNA C/D box 24 (RNU24) quantitation were performed using the Custom TaqMan Small RNA Assays and TaqMan Fast Universal PCR Master Mix No AmpErase UNG (Applied Biosystems, CA, USA) according to the manufacturers' instructions. The miRNA quantitation in Paper III was performed by using the TaqMan Gene Expression Master Mix together with the respective TaqMan MicroRNA Assays (both from Applied Biosystems, CA, USA). All the samples were run on the Step-One Real-Time PCR instrument (Thermo Fisher Scientific, MA, USA). The cycle threshold (Ct) values were obtained from the StepOne Software (Applied Biosystems, CA, USA); the PCR efficiency of each reaction was calculated with LinRegPCR program[406] or obtained from the StepOne Software. ΔΔCt (Paper I) or efficiency (E)^-Ct (relative to cel-miR-39 RNA extraction control level; Paper III) methods were used to express the levels of the targets under study.

7.5 Next generation sequencing of small RNAs

7.5.1 Sample preparation and sequencing

Paper II and III include small RNA sequencing of cells, vesicles and non-vesicular secretome. The RNA from the aforementioned samples was extracted by following a standard phenol-chloroform extraction protocol using either Trizol or Trizol LS solutions (both from Thermo Fisher Scientific, MA, USA). Improved RNA precipitation was gained by adding 2 µl of PolyAcryl Carrier PC 152 polymer (Molecular Research Center Inc., OH, USA) per reaction. The RNA integrity of the cell samples was verified on Bioanalyzer RNA 6000 Pico Total RNA Kit (both Agilent Technologies, UK) and the RNA concentration for all samples was measured with the Qubit 2.0 Fluorometer by using the Qubit RNA HS Assay Kit (Thermo Fisher Scientific, MA, USA). Either 60 ng (Paper II) or 250 ng (Paper III) of total RNA was subjected to small RNA library preparation by using the NEBNext Multiplex Small RNA Library Prep for Illumina (NEB, MA, USA) kit according to the manufacturer's instructions. The barcoded samples were size selected on a 6 % Novex TBE PAGE gel (Thermo Fisher Scientific, MA, USA), the fragments corresponding to microRNA range were cut out and subjected to purification with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany). Thereafter, the products were quantified by using the KAPA Library Quantification Kit (Kapa Biosystems, UK) and pooled at equimolar ratio. In Paper II, two libraries (technical replicates) were generated in parallel, each eventually containing a pool of 12 barcoded samples. In Paper III, 18 barcoded samples (biological replicates) were pooled. In both cases, the readymade libraries were checked on the High Sensitivity D1000 ScreenTape (Agilent Technologies, UK) and quantified using the KAPA Library Quantification Kit (Kapa Biosystems, UK) to enable precise loading of the flow cell. The clusters were generated by using the cBot and sequenced one replicate per lane on either one

(Paper II) or two (Paper III) flow cells on the HiSeq2500 (Illumina Inc., CA, USA) with a 1x51 setup in RapidRun mode.

7.5.2 Data analysis

Small RNA sequencing data was analysed in a similar manner for both Paper II and Paper III. Briefly, raw sequencing reads were quality controlled by FastQC [407] analysis and subjected to adapter removal by Cutadapt/1.9.1 [408]. All reads with an adapter and a length of 17-35 bases (filtering with BBMap release 35.40 [409]) were subjected to subsequent mapping on the Ensembl 38.85 releases of the mouse and the human genome by using Bowtie1 (release 0.12.6) [410] in -v1 alignment mode and best alignment stratum reporting option. Annotation was performed in a stepwise manner with HTSeq (release 0.6.1) [411] in stranded mode by following a stepwise annotation procedure allowing the discrimination of 'small RNAs', followed by 'ribosomal RNA' and 'other RNAs'. Gene biotype classification followed the classification details in Vega Genome Browser release 68; details of the included RNA biotypes can be found in Paper II. The annotations of different RNA biotypes were retrieved from miRBase release 21 [412], Ensembl 38.85 [413], piRNAbank [414] and UCSC Table Browser hg38/mm10 entries [415].

For data visualization, MultiQC v1.3 [416] and the online analysis software Morpheus (available from the Broad Institute; https://software.broadinstitute.org/morpheus; Paper II) as well as Multiple Experiment Viewer (Version 4.9.0) [417] and R-studio software [418] (R version 3.4.2; Paper III) were used. Differential expression analysis of miRNAs was performed by using the R package DESeq2 [419]. All statistical analyses (except for differential expression statistics) were performed using GraphPad Prism Version 6 or 7 (GraphPad Software, Inc., CA, USA).

7.6 Proteomic analysis

The proteomic analysis of EVs in Paper II exploited liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously [114]. The analysis covering Gene Ontology (GO) term enrichment and -overrepresentation was performed by using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) software [420]. In addition, the study included an in-depth analysis of the 'RNA binding' proteins (GO:0003723), obtained via the QuickGO browser (http://www.ebi.ac.uk/QuickGO/) and Vesiclepedia [421] database. The list of 'miRNA related' proteins was created by manual curation of the 'RNA binding' proteins. All proteomic analysis was based on unique protein identifiers, thereby taking into account different protein isoforms.

7.7 *In vivo* techniques

7.7.1 *In vivo* delivery of nucleic acid complexes

In Paper I, in order to evaluate the potency of LF2000 lipoplexes, 5 µg of firefly luciferase expressing plasmid (PT2/C-fluc plasmid; Addgene plasmid 20203; Addgene, MA, USA) was used either on its own, or complexed with 12.5 µg freeze-thawed or non-frozen LF2000. The complexes were formed in OptiMEM serum free medium and a total volume of 50 µl was used for contralateral intramuscular (i.m.) injections in mice. The luminescence was imaged 24 hours post-injection using the IVIS Imaging System (PerkinElmer, MA, USA).

7.7.2 Tracking and tissue distribution of EVs

Paper IV assessed the tissue distribution of EVs from different cell lines using intravenous, intraperitoneal or subcutaneous injection to NMRI or C57BL/6 mice. In order to track the EVs *in vivo*, a near-infrared fluorescence dye, DiR (1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide; D12731, Invitrogen, Life Technologies) was used. To label the EVs, the filtered CM was co-incubated in the presence of 1 µM of DiR at room temperature for 15 minutes. Thereafter, the EVs were purified by following the EV enrichment protocol by UC, as described above. The same procedure was employed for unconditioned medium, serving as the control to determine the extent of unspecific tissue distribution. The biodistribution of the EVs was evaluated with the IVIS Imaging System (PerkinElmer, MA, USA) with or without organ dissection. The dosage of EVs was based on NTA particle quantification and was optimized within the study to 1E10 particles/gram of body weight in order to ensure sufficient fluorescence detection and minimal signal saturation in the investigated organs. The tissue distribution of CD63-eGFP positive EVs in explanted organs was also evaluated immunohistochemically.

RESULTS AND DISCUSSION

8 Paper I

Paper I outlines the effect of cryo-manipulation of a cationic lipid-based formulation, LF2000, which, after being frozen and thawed, showed orders of magnitude higher nucleic acid delivery *in vitro* and *in vivo* than the non-frozen counterpart.

The preliminary observations of the increased potency were made with luciferase-encoding plasmid delivery. As a result, a log-scale improvement in nucleic acid transfection efficiency throughout eight different cell lines, including hard-to-transfect cells such as human fibroblasts and mouse myotubes, was observed. The effect was most prominent at lower nucleic acid doses and was witnessed with different batches of LF2000. To better quantify the cell viability as well as the number of cells with successful plasmid delivery and expression, we employed flow cytometry analysis. These results further confirmed the significantly better performance of freeze-thawed LF2000 over non-frozen with up to 45% increase in the number of transfected cells. A decrease in cell viability with flow cytometry as well as cell proliferation assay (WST-1) was observed at the highest plasmid concentration for the freeze-thawed formulation. Yet, this was valid only for Neuro2a cells.

To exclude the possibility that the potency was a result of a specific plasmid, expression cassette and/or type of promoter used, the non-frozen and freeze-thawed LF2000 were tested in a splice-correction assay using U7 snRNA construct [422] or different splice-correcting oligonucleotides in a HeLa Luc 705 cell line [405]. Consistent with the previous results, cells transfected with freeze-thawed LF2000 displayed significantly higher luciferase signals than those transfected with non-frozen reagent, confirming that the effect was consistent regardless of the plasmid organization or the chemical composition of the nucleic acid.

As it is known that conditions optimized for *in vitro* transfections are often not applicable to efficient *in vivo* delivery [423], we further evaluated whether freeze-thawing of LF2000 has an impact on nucleic acid delivery *in vivo*. Indeed, i.m. injection in mice of a luciferase plasmid complexed with non-frozen or cryo-manipulated LF2000 confirmed the *in vitro* results where a log-scale higher luminescence in the muscle treated with freeze-thawed reagent was observed.

We next became interested whether the effect could persist over a longer period of time and/or could be obtained by other ways of cryo-manipulation. After overnight freezing, thawing and storage for two weeks+4ºC the reagent still showed improved transfection efficiency over non-frozen LF2000. Repeated freeze-thawing cycles and snap-freezing in liquid nitrogen also exhibited higher transfection efficiencies than the non-frozen counterpart. Yet, the best overall potency was seen with the reagent that was used immediately after overnight freezing.

By studying the physical characteristics of non-frozen and freeze-thawed LF2000 complexes, we saw that the latter formed a much more heterogeneous population of particles, with a

slightly smaller size, yet with no apparent differences in their morphology or nucleic acid release potency. Nonetheless, by exploiting TIRF microscopy, we observed that the freeze-thawed complexes dispersed more readily on a glass surface and sedimented to a higher degree than the non-frozen LF2000. These properties are likely to underlie the increased cellular uptake of freeze-thawed LF2000 lipoplexes as well as elevated cellular activity.

To find further explanations to the phenomena, we explored the effect of freeze-thawing on a handful of other lipid-based and non-lipid based transfection reagents. As a result, we saw a similar increase in transfection efficiency for Lipofectamine RNAiMAX reagent, allowing us to speculate that the outstanding transfection efficiency relates to the specific structure or composition of both Lipofectamine products.

In conclusion, this study reveals that simple cryo-manipulation is able to significantly increase the nucleic acid delivery potency of a commercial cationic lipofection reagent and is likely to be applicable for an even wider range of lipid-based delivery agents. Importantly, our results illustrate that freeze-thawing allows to drastically reduce the amount of transfection reagent needed for cellular transfection, while retaining the desired activity.

9 Paper II

EVs mediate their native biological effects by transferring or displaying their cargo to target cells. While certain broad-spectrum EV mediated effects reflect their protein cargo composition, others have been attributed to individual EV-loaded molecules such as specific miRNAs [338,424]. In this work, we investigated the cell line dependent differences and interplay of small RNA and protein cargo in unmodified EVs.

Firstly, we performed small RNA sequencing on UC-purified EVs as well as their source cells of human (HEK293T, RD4) and mouse (C2C12, Neuro2a and C17.2) origin. By size-selecting the libraries and length-restricting data analysis to sequences of 17–35 nucleotides in length, we were able to see that the majority (~80%) of the cellular RNA did represent "small RNA" sequences, of which a great majority (73-93%) represented miRNAs, confirming the reliability of the applied methodology. Across different cell lines, the EVs had considerably less miRNA and more sequences derived from piwi-like RNA (piRNA) loci than their source cells. Hierarchical clustering analysis of miRNA signatures revealed that all the EV samples clustered together with their parental cells as opposed to EVs from other cell sources. Though the number and relative expression of EV miRNA sequences was in good correlation with the cellular background, for piRNA sequences, no clear correlation between the vesicular and cellular expression level was found. Due the lack of proof of the association of piRNA sequences with piRNA proteins, together with the evidence of a relative scarcity of characteristic piRNA features (e.g. length of 27-35 nucleotides, 5' uracil bias), these RNA stretches rather represent sequences derived from piRNA loci than *bona fide* piRNAs.

Though, as mentioned earlier, the majority of cellular sequences represented "small RNAs", it is noteworthy to mention that the "small RNA" content of EV samples was highly variable across the tested cell types. On average only ~22% of the sequences were derived from "small RNA" loci, while the reads derived from rRNA loci were more prominent and covered 36-94 % of all annotations in EVs. In addition, both cells and EVs had a large number of tRNA and Y RNA sequences, corroborating with earlier EV-based studies also employing short read-length sequencing [212,217,238,425]. For all RNA categories ("small RNAs", "rRNAs" as well as "other RNAs"), a small number of highly abundant sequences covered the bulk RNA content within each category, illustrating the relatively low diversity of the RNA mass. The prominence of individual RNA species was explicitly evident from the list of overall top-ranking RNA genes, where the EV samples were dominated by highly abundant "rRNA" and "other rRNA" sequences, whereas the cells samples were rather enriched in miRNAs.

Next, we set out to explore whether the EV proteome, particularly the repertoire of RNA-binding proteins would correspond to our observations about the RNA species in EVs. This part of the study was performed on HEK293T and C2C12 EVs given their disparate 'small RNA' (and thus also miRNA) content. In both proteomes, we detected ~2000 proteins, of which ~60% overlapped with entries in the Vesiclepedia database [421]. Similarly to transcriptomic results in which a relatively small number of RNA sequences contributed to a large proportion of total detected RNA reads, a small number of highly abundant proteins (~200 identifications) covered the bulk content (~75%) of both EV proteomes. Also, in line with the finding of a high abundance of ribosomal, coding and tRNA fragments in the sequencing, we discovered high levels of rRNA-, poly(A)- and tRNA binding proteins in the proteomic datasets of both EV types. GO analysis revealed that ~20-30% of the identified proteins in EVs were "RNA binding" (GO:0003723), correlating well with the mean reported frequency (21 %) of RNA binding proteins in Vesiclepedia database [421]. Similarly to the results of the bulk proteome, the "RNA binding" proteins that contributed most to the EV proteome described poly(A)- and rRNA binding proteins, double- and single-stranded RNA binding proteins as well as translation-related protein sets.

In order to further understand which RNA-binding protein classes are represented in the data sets and how these correspond to the small RNA sequencing results, we created a custom curated GO list of "miRNA related" proteins. Thereby, we were able to define that ~1 % of the HEK293T and C2C12 EV proteins were relevant to the molecular function, biological processes or cellular components of miRNAs. Most of them represented proteins with very low expression level, resulting in their contribution to the total EV protein mass by only ~0.9 % for both samples. Though C2C12 EVs were substantially richer in miRNAs than HEK293T EVs, we were unable to detect any major differences in the miRNA-related proteome, precluding us from further speculations of whether specific miRNA binding proteins drive the miRNA sorting into EVs. In addition, even though EVs showed a high number of piRNA sequences, we were unable to detect any "piRNA binding" proteins,

aligning well with our observation that the sequences annotated as piRNAs in this study rather represent reads mapping to piRNA loci.

In conclusion, this study investigated the vesicular RNA and protein cargo composition and interplay in a handful of cell types. The present data is particularly useful for future work in unravelling the biological mechanisms underlying vesicular RNA and protein sorting and serves as an important guide in developing EVs as carriers for therapeutic RNA interventions.

10 Paper III

Proceeding from the results of Paper II, we became increasingly interested in exploring the wider composition of the cellular secretome and decided to concentrate more on the specific miRNA profiles. To look into the composition of the EV secretome as well as investigate the non-EV material, we decided to exploit SEC [114] enabling to discriminate and fractionate both secretory portions.

Owing to earlier reports [105,107], it is known that a large amount of miRNA is secreted outside of EVs. To investigate by which extent miRNAs of interest get released to the extracellular space, we transiently overexpressed pri-let-7a and pri-let-7b in HEK293T cells, and quantified respective mature miRNA levels in the secretome. As a result, we could see that upon overexpression the amount of non-EV miRNA exceeds tens of folds the miRNA level seen in the EVs. Though similar evidence has already been seen by centrifugation-based studies [426,427], we hereby provided additional insights and profiled the miRNA abundance across the secretome.

Given the log-scale differences in the miRNA amount between the EV and non-EV fractions, we became interested whether the global pool of miRNAs follows a similar secretion profile. The subsequent small RNA sequencing analysis was performed on the HEK293T cells and hTERT-MSCs as well as their EV and non-EV secretory fractions, separated identically to the preliminary overexpression studies.

When looking in detail into the small RNA secretome, the bulk secretory material consisted predominantly of miRNAs and piRNA-like sequences. The total miRNA efflux to the extracellular environment was comparable for EVs and non-EV samples. Also, we observed that the expression levels of EV-, non-EV and cellular miRNAs were well correlated, apart from some DE miRNAs in the EVs and non-EV fraction, which exceeded several fold the levels in their source cells. Irrespectively, a vast majority of all the DE hits represented miRNAs with relatively low expression in both the source cells as well as in the secretory fractions, fitting with the correlation of the samples' miRNA levels, indicating that the bulk of the secretome does rather resemble the cell of origin and suggesting that the majority of the miRNA follows a passive secretion mechanism.

To reflect on the total miRNA content of the secretome, we decided to quantify the total amount of candidate miRNAs in the EV and non-EV fractions by using RT-qPCR from an

equal volume of starting material. As a result, we could see that the total amount of non-vesicular miRNA exceeded tens to hundreds of folds the basal miRNA amount in EVs. Taking into account the distinct miRNA quantities at their basal level in both secretory fractions, above 90% of the total extracellular miRNA got secreted to the non-vesicular fraction, coinciding with our preliminary observations of overexpressed non-EV miRNA levels.

In summary, this study outlines that the bulk miRNA content in cells, EV and non-EV fraction is highly alike, whereas the majority of DE miRNAs represent low-abundant molecules and miRNAs both at their overexpressed and basal level are predominantly found in the non-EV portion of the secretome. This information is valuable in order to gain a thorough understanding of short RNA sorting mechanisms into EVs and thereby progress the employment of EVs as therapeutic nucleic acid carriers.

11 Paper IV

In order to develop EVs as carriers for therapeutic RNA interventions, it is of utmost importance to have a clear understanding of their body-wide distribution. Thus, Paper IV was brought about to gain a comprehensive overview of the biodistribution of exogenous EVs, its dependence on the administration route, cell source, dosing as well as on the potential of targeted tissue distribution.

Firstly, in order to track the EVs *in vivo* we set out to validate the labelling of EVs incorporating the near-infrared lipophilic dye (DiR). Lipophilic DiR dyes are known to possess a low level of autofluorescence, give high fluorescence output once incorporated in membranes and offer good tissue penetrance owing to their near-infrared spectrum. Yet, the incorporation of unbound dye into cell membranes as well as its transfer between neighboring cells results in unspecific fluorescence events [428], emphasizing the need of proper background controls. To evaluate the performance of the labelling strategy, the labelling of DiR EVs was evaluated against a free DiR dye control on a density gradient separation. As expected, the free DiR dye displayed a lower buoyant density and fluorescence than the DiR labelled EV samples, indicating the presence of free dye, devoid of lipid membranes. The density and fluorescence profile of DiR labelled EVs across the gradient as well as the co-localization of EV marker ALIX indicated successful labelling. After UC purifying the DiR labelled unconditioned medium and additional monitoring of free DiR dye *in vivo*, we could confirm that any excess dye would be lost during EV isolation, allowing us to proceed with the chosen labelling strategy.

In order to see whether and how the dose could affect the distribution of exogenous EVs, we conducted a dose titration study; 0.25×10^{10} - 1.5×10^{10} particles per gram (p/g) of body weight were intravenously (i.v.) injected into mice, followed by organ harvesting 24 hours post-injection. Notably, even though at increasing doses the overall tissue distribution pattern did not change, the relative fluorescence values in the liver were decreased. This was

interpreted as a potential saturation of the MPS, leading us to choose the intermediate dose of 1.0×10^{10} p/g for subsequent experiments.

Due to employment of lipophilic EV labelling strategy, we also evaluated the kinetics of EV biodistribution in order to rule out the tracking of free- as opposed to EV-bound DiR dye and found that the overall biodistribution up until 24 hours remained largely unchanged. Yet, we did observe an initial pulmonary accumulation of EVs at 5 minutes post-injection, plausibly representing the primary exposure of administered EVs to the capillaries of the lung. At the 48 hours' time point we observed increased changes in the tissue distribution profile (e.g. high accumulation in pancreas), potentially representing EV redistribution events, a later EV uptake phase or an artefact due to the long half-life of the dye [240].

The most common administration route for EV-based *in vivo* studies is i.v. injection. Yet, it is important to assess how different administration routes affect the distribution of EVs as well as whether different routes can be used to boost a desired therapeutic outcome. To estimate the effect of EV administration routes, we investigated the biodistribution differences between i.v., intraperitoneally (i.p.) and subcutaneously (s.c.) injected vesicles. Thereby, we could see that the i.v. injected EVs accumulated significantly more in the liver and spleen as opposed to i.p. or s.c. injected EVs. The s.c. route also resulted in significantly lower fluorescence values in the *ex vivo* imaged organs, plausibly indicating to the retention of EVs in the adipose tissue. These results highlight the importance of the choice of EV administration route in order to either obtain a favourable biodistribution pattern or a desired pharmacokinetic profile.

The intrinsic tissue tropism of EVs has been a matter of intense debate in the field. To explore the organotropic preferences, we investigated the biodistribution patterns of EVs derived from 3 mouse (C2C12; B16F10; primary immature bone-marrow derived DCs), 1 rat (OLN-93) and 2 human cell types (HEK293T; primary human MSCs) 24 hours post i.v. injection. Generally, the highest accumulation of EVs was seen in liver, followed by spleen and lungs. Yet, the distribution pattern of DC EVs deviated significantly from this accumulation profile, exhibiting strong fluorescence from the spleen and weak signal from the liver as compared to other tested EV sources. Considering earlier reports dissecting the integrin-dependent EV organotropism [248], we hypothesize that the observed distribution pattern could also be caused by a unique repertoire of molecules displayed on the surface of DC EVs. In light of a great potential of EV tropism for targeted therapies, further studies are needed to unravel the extent and specific mechanisms of the phenomenon.

Lastly, to evaluate the impact of EV surface engineering on targeted tissue delivery, we employed DC-derived EVs expressing a chimeric Lamp2b-RVG on their surface. RVG is known to bind to the acetylcholine receptors [429] and enhance brain targeting of EVs, as evidenced by earlier studies [79]. Indeed, a small, but significant 2-fold increase in the brain accumulation of targeted EVs was detected thereby leaving the overall biodistribution of EVs largely unaltered. This exemplifies the sensitivity of the EV labelling strategy employed

throughout the study as well as indicates the potential of using targeting moieties on the EV surface.

In conclusion, this study showed that DiR labelling is a suitable methodology for tracking EVs in *in vivo* assays with high specificity and sensitivity. In addition, even though the majority of EVs accumulate in liver and spleen, the distribution pattern could be affected by the cell source, administration route, dose and the presence of targeting moieties on the EVs.

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