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ENGINEERED EXTRACELLULAR VESICLES FOR BIOMEDICAL APPLICATIONS

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Cover illustration: Coloured scanning electron micrograph (SEM) of a cultured 293T cell releasing large numbers of exosomes. Photo by Steve Gschmeissner. Image licenced from Sciencephotolibrary for thesis use only.

Engineered Extracellular Vesicles for Biomedical Applications
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my family and friends

ABSTRACT

Nature's very own nanoparticle, Extracellular vesicles (EVs), are lipid membrane-enclosed vesicles encapsulated with diverse biomolecules and are actively secreted by all cell types for intercellular communication. The unique properties of EVs, such as stability in circulation, biocompatibility, immune tolerance, and the ability to cross biological barriers, render EVs a next-generation drug delivery tool. Therapeutic EV research has seen tremendous development in the past decade, from *in vitro* studies towards pre-clinical models to various clinical trials. Even so, the road towards successful clinical translation has faced various hurdles primarily due to the lack of technology to address the knowledge gap in EV biology. Hence, this thesis is focused on addressing some of these critical challenges and exploring novel biomedical applications for EVs.

EVs are considered as essential mediators in physiology and disease pathology. However, to elucidate their important role in pathophysiology or as therapeutics, sensitive tools for visualising them are much needed. Here, in paper I, we have developed a sensitive bioluminescent labelling system for tracking EVs *in vitro* and *in vivo*. By genetically modifying the producer cells with EV-associated tetraspanins-fusions, we could efficiently load luciferase enzymes (Nanoluciferase and Thermoluciferase) into EVs. Utilising the Nanoluciferase labelling system, we could detect as low as 5000 EVs in a solution, and the naked eye could visualise the luminescence generated from these EVs. With this level of sensitivity, we explored various *in vivo* applications and observed that exogenous EVs are rapidly distributed throughout the body, primarily to the liver, lung, and spleen. In addition, we identified that EV subpopulations differ in their *in vivo* biodistribution profile. In summary, this system allows for highly sensitive detection of EVs *in vivo* and reflects the true fate of EVs.

Despite tremendous advancement in understanding EV biology or engineering, techniques to surface engineer EVs with large protein biotherapeutics without altering their innate properties are largely lacking. Here in paper II, we developed a novel surface display technology for EVs, which allows for efficient display of several membrane proteins on the EV surface simultaneously. Using this platform, we decorated EVs' surface with cytokine receptors that can decoy pro-inflammatory cytokines such as TNF-α or IL-6/sIL-6R complexes. These cytokine decoy EVs were more active than a clinically approved biologic against TNF-α *in vitro*. Importantly, these cytokine decoy EVs ameliorated the disease phenotype in three different mice inflammation models, including neuroinflammation. In paper III, we have applied interleukin 6 signal transducer (IL-6ST) decoy EVs to tackle inflammation in muscle pathologies to enhance the muscle regeneration process. Using decoy EVs as a therapeutic intervention in mdx mice mimicking Duchene Muscular Dystrophy (DMD), we could achieve significant downregulation of phosphorylation of the pro-inflammatory transcription factor STAT3 in muscles.

In conclusion, the tools developed in this thesis, from highly sensitive detection of EV subtype to efficient display of biotherapeutics cargo on EV surfaces, holds great future potential and applicability in numerous biomedical applications of EVs.

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LIST OF ABBREVIATIONS

AAVs	Adeno-Associated Viruses
ADAM	A Disintegrin and Metalloproteinase
ALIX	ALG-2-Interacting Protein X
ARRD	Arrestin-Domain-Containing Protein 1
BAR	Bin, Amphiphysin and Rvs
BASP-1	Brain Acid Soluble Protein 1
CD	Cluster of Differentiation
CM	Conditioned Medium
CNS	Central Nervous System
CPP	Cell Penetrating Peptide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAF	Decay-Accelerating Factor
DC	Dendritic Cell
DMD	Duchenne Muscular Dystrophy
EAE	Experimental Autoimmune Encephalomyelitis
EGFO	Enhanced Green Fluorescent Protein
EGFR	Epidermal Growth Factor Receptor
EM	Electron Microscopy
ENV	Envelope Protein
ER	Endoplasmic Reticulum
ERV	Endogenous Retroviruses
ESCRT	Endosomal Sorting Complex Required for Transport
EV	Extracellular Vesicle
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
GMP	Good Manufacturing Practice
GPCR	G protein Coupled Receptor
GPI	Glycosylphosphatidylinositol
HD-PTP	His Domain Phosphotyrosine Phosphatase
HDL	High-Density Lipoprotein
HEK293	Human Embryonic Kidney 293
HIV	Human Immunodeficiency Virus
HMPAO	Hexamethylpropyleneamine oxime

HNRNPK	Heterogeneous Nuclear Ribonucleoprotein K
I-BAR	Inverse-Bin, Amphiphysin and Rvs
IBB	Iodide-bromine Balneotherapy
ICAM-1	Intercellular Adhesion Molecule
IgSF8	Immunoglobulin Superfamily Member 8
IL-6	Interleukin-6
IL-6ST	Interleukin-6 Signal Transducer
ILV	Intraluminal Vesicle
KRAS	Kirsten Rat Sarcoma Virus
Lamp2b	Lysosome-Associated Membrane Protein 2b Latent Membrane Protein 1
LBPA	Lysobisphosphatidic acid
LDL	Low Density Lipoprotein
LNP	Lipid Nanoparticle
LPS	Lipopolysaccharide
MAC-IP	MAC-inhibitory Protein
MFGE8	Milk fat globule-EGF factor 8
MHC	Major Histocompatibility Complex
miRNA	micro-RNA
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MSC	Mesenchymal Stromal Cells
MV	Microvesicle
MVB	Multivesicular Body
MWCO	Molecular Weight Cut-Off
NGS	Next Generation Sequencing
NHS	N-Hydroxysuccinimide
NK	Natural Killer
NTA	Nanoparticle Tracking Analysis
PA	Phosphatidic acids
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PD-L1	Programmed death-ligand 1
PEG	Polyethylene Glycol
PET	Positron emission tomography

PI	Phosphatidylinositol
piRNA	Piwi Interacting RNA
PS	Phosphatidylserine
PTEN	Phosphatase and Tensin homolog
PTM	Post-Translational Modifications
RBC	Red Blood Cell
RES	Reticuloendothelial System
RFP	Red Fluorescence Protein
RNP	Ribonucleoprotein
rRNA	Ribosomal RNA
RVG	Rabies Viral Glycoprotein
SEAP	Secreted Alkaline Phosphatase
SEC	Size Exclusion Chromatography
SNAP	Synaptosomal-Associated Protein
SNAP23	Synaptosomal-Associated Protein 23
SNARE	Soluble NSF Attachment Protein Receptor
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
SPION	Superparamagnetic Iron Oxide Nanoparticles
SYNCRIP	Synaptotagmin-Binding Cytoplasmic RNA-Interacting Protein
TEM	Transmission Electron Microscopy
TFF	Tangential Flow Filtration
TGFB	Transforming Growth Factor Beta
TNBS	2,4,6-Trinitrobenzenesulfonic acid
TNF	Tumour Necrosis Factor
tRNA	Transfer RNA
TSG101	Tumour Susceptibility Gene 101
UA	Uranyl Acetate
UC	Ultracentrifugation
UF	Ultrafiltration
VAMP	Vesicle-Associated Membrane Protein
VAMP7	Vesicle-Associated Membrane Protein 7
VLDL	Very Low-density Lipoprotein
VLP	Virus-Like Particles

VPS4	Vacuolar Protein Sorting-Associated Protein 4
VTA-1	Vacuolar Protein Sorting-Associated Protein VTA1 Homolog
WB	Western Blot
WBC	White Blood Cells
YBX1	Y-Box Protein 1

1 INTRODUCTION

1.1 HISTORY OF THE EXTRACELLULAR VESICLE FIELD

Prokaryotes to eukaryotes have evolved diverse mechanisms to exchange informative signals to achieve sustainability in a multicellular environment. Recent advances in science have led to the discovery of vesicle structures released by cells, which play a critical role in intercellular communication by transferring bioactive molecules from one cell to another[1,2]. The existence of extracellular vesicles (EVs) was first showcased by Chargaff and West while studying the effect of high-speed centrifugation of plasma on coagulation time [3]. They observed that reddish-brown translucent pellets sedimenting at high-speed centrifugation possessed clotting properties. Building on this, Wolf in 1967 further investigated the breakdown products from blood corpuscles by electron microscopy and referred to these small membranous particles originating from platelets as "platelet dust"[4].

In the following years, seminal work by Aaronson *et al.* and Dalton *et al.* showed that these vesicles are made of standard cell components and were neither virus-like particles nor arising from any artefacts related to electron microscopy (EM)[5]. Later studies further strengthened these observations that EVs are not cellular debris or viruses but are bioactive lipid enclosed vesicles derived from cellular compartments[6,7]. In 1981, outstanding work by Trams *et al.* laid the foundation of the term exosomes. In their work, they reported irregularly shaped vesicles of a size range of 500-1000 nm formed by membrane exfoliation from normal and neoplastic cells. Importantly, they also observed smaller 40 nm vesicles in large microvesicle fractions similar to multivesicular bodies (MVB)[8], which are formed by inward budding of the endosomal membrane. Their study suggested that these plasma membrane vesicles should be called exosomes. Later that decade, milestone work by R.M Johnstone and colleagues on reticulocytes made a significant contribution to the knowledge on the biological role of EVs. In their work, they observed the formation of MVB like structures, and upon fusion with the plasma membrane, it led to the extracellular release of vesicles carrying transferrin receptors. The authors described this as a method to recycle proteins for their maturation[9–11].

Similar MVB structures carrying MHC molecules were reported in other cells such as B lymphocytes[12] and dendritic cells[13] and, upon fusion with the plasma membrane, led to the secretion of EVs, which could induce T cell response. Despite tremendous advancement in the knowledge of EVs, a majority of the scientific community was sceptical about EVs, and they were largely considered garbage bags. However, EVs made a remarkable comeback in the late 2000s when three separate studies reported that EVs carry nucleic acids and can horizontally transfer them from one cell to another[14–16]. Today, EVs are considered to play an important role in physiology and disease pathology. The field has expanded rapidly to this day, which is evident with an increasing number of publications every year (Figure 1). From

the initial discovery of exosomes and other EVs, we are now at the point where the field is trying to delineate the heterogeneity within each class of EVs.

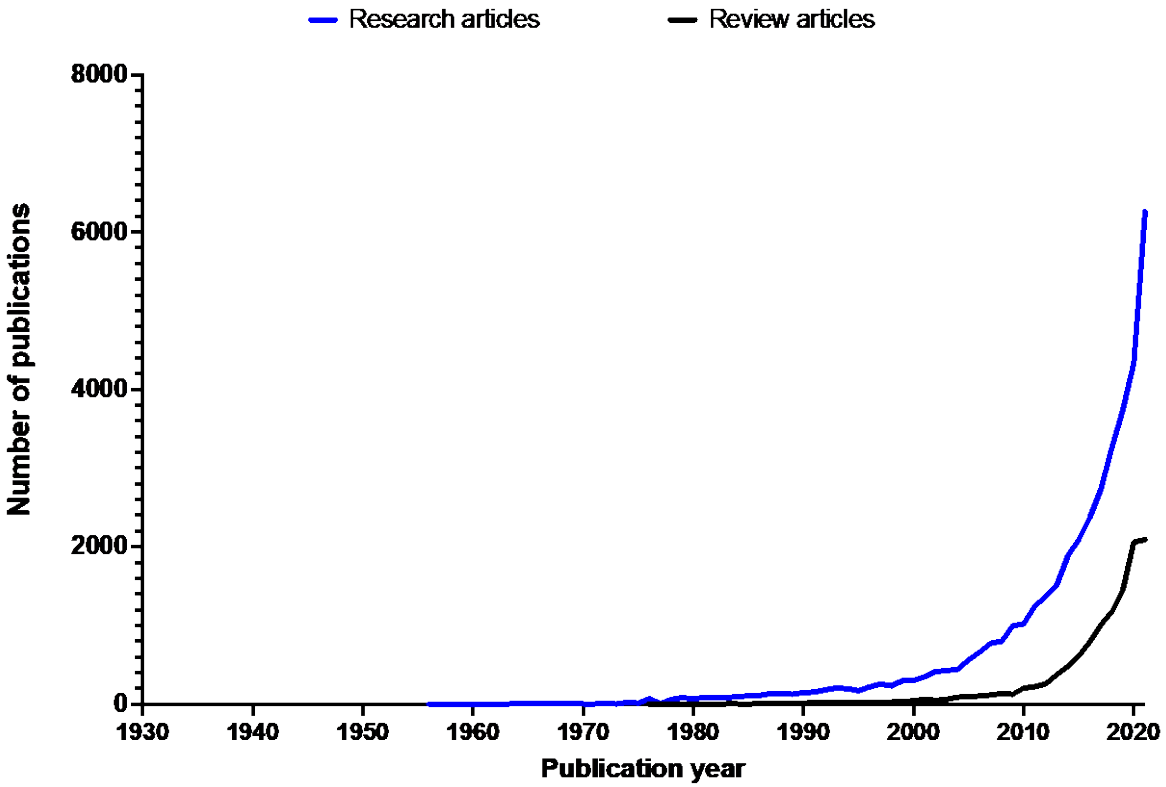

Figure 1. The total number of publications, including research articles and review articles (as of 1 Dec 2021) identified on PubMed from 1970 onwards using any of the following terms: exosomes, extracellular vesicles, microvesicles.

1.2 EXTRACELLULAR VESICLES CLASSIFICATION

The nomenclature of cell-secreted vesicles has been chaotic in the EV research field. Due to the lack of consensus among EV researchers, a variety of classification systems have been devised to address the heterogeneity of EVs. Some classify based on the function of EVs (e.g. "tolerosomes" for vesicles involved in immunological tolerance against dietary antigens [17]) or based on producer cells (e.g. "dust from platelets" [18] and "prostasomes" by prostate epithelium [19]) or their size. Albeit this nomenclature could be beneficial in some settings as it reflects the function of EVs, it lacks general relevance. Instead, a nomenclature based on biogenesis offers broader applicability and accommodates EVs' heterogeneity much better than other systems. Furthermore, to clarify the confusion on nomenclature Gyorgy *et al.* in 2011[20], El Andaloussi in 2013[21] and Gould and Raposo in 2013[22] emphasised how different EV terms are often misused and using biogenesis as a means for classifying different EV types may resolve the confusion. Similar thoughts were also portrayed by the international society of extracellular vesicles in their position papers[24,25].

Using biogenesis as a classifier for EVs, the term EV can be subdivided into various classes of vesicles, with a vast spectrum of sizes ranging from 30 nm to 10 µm. Exomeres [26] represent the smallest known population of extracellular vesicles or nanoparticle (as they lack membrane), followed by exosomes, microvesicles (MVs), large oncosome vesicles [27], migrasomes [28], mitovesicles, and apoptotic bodies (Figure 2). Recently discovered, exomeres are non-membranous nanoparticles that are 40 to 70 nm in diameter. These nanoparticles are composed of proteins, nucleic acids, and lipids[29]. Importantly their biological role and biogenesis pathways are relatively unknown, but they are enriched in various signalling ligands such as EGFR and metabolic enzymes[30,31]. The second class of EVs is exosomes which are 50-200 nm in diameter and originate from the endolysosomal pathway[2,32]. These are followed by MVs (also known as ectosomes[33]), which are variable in size (0.1-1µm in diameter) and shed directly from the plasma membrane[34,35]. Similarly, large oncosomes, which are 1-10µM in diameter, originate from the plasma membrane during amoeboid migration of metastatic cancer cells[27,36]. The latest addition to the EV troop is migrasomes, and they are formed on the tip or intersections of retraction fibres protruding out from migrating cells. They are 0.5 to 3µm in diameter and contain multiple small vesicles of 50-100 nm each, which are released upon the rupture of retraction fibres. In addition, to migrasomes, double-membrane vesicles which are 100-300 nm in diameter, have been identified in mouse and human brains. Although their biological role is unclear, they are enriched in mitochondrial components, thus accounting for their name “mitovesicles” [37]. The last known class of EVs is apoptotic bodies that bleb from apoptotic cells and can vary in size from 0.8-5µm[38]. This thesis will focus primarily on the three most common EV types; exosomes, MVs, and apoptotic bodies.

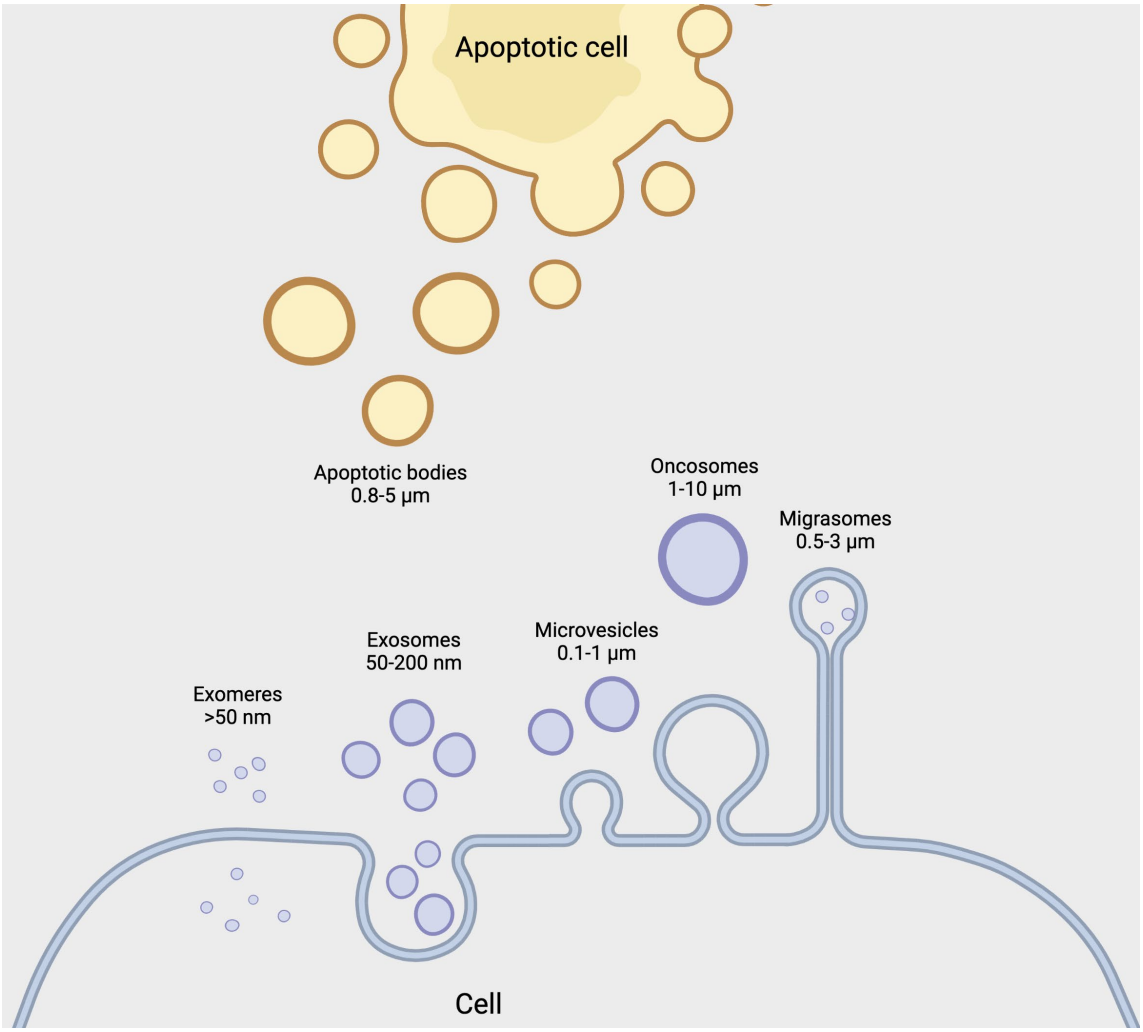

Figure 2. Classification of extracellular vesicles. Different types of vesicles secreted by cells based on their biogenesis pathway.

1.3 BIOGENESIS OF EV

Different classes of EVs have different modes of biogenesis. Exosomes are generated through the endolysosomal system, whereas MVs are formed by outward budding of the cell membrane (Figure 3), however exosome biogenesis has also been shown to occur directly at the plasma membrane[39,40]. Since the currently known biogenesis machinery of exosomes and MVs overlap to some degree, it is hard to distinguish between these two vesicle populations[41–44]. However, with the technological advancement over the years, the distinct mechanism involved in their generation are being unravelled. This section will summarise the known mechanisms involved in the biogenesis of exosomes and MVs.

1.3.1 Exosome biogenesis

The biogenesis of exosomes is a complex biological process that involves multiple signalling pathways and regulators. From the formation to the release of exosomes, the process can be

subdivided into three main steps: 1) The formation of the MVBs, 2) Transport of MVBs to the plasma membrane and 3) Fusion of MVBs with the plasma membrane.

1.3.1.1 MVB biogenesis

The first step towards exosome biogenesis is the formation of early endosomes, these intracellular vesicles are formed by endocytosis, which is a membrane invagination process occurring at the cell surface. In certain instances, vesicles arising from trans-Golgi network budding can also additionally fuse with the early endosomes to facilitate cargo loading into the exosomes. Upon maturation, early endosomes transition into late endosomes via conversion of Rab GTPases from Rab 5 to Rab 7 or transport carriers[45,46]. During the maturation process of early endosomes towards late endosomes, invagination of the endosomal membrane drives the formation of intraluminal vesicles (ILVs). This process is regulated by ESCRT-0,-I,-II and -III assembly and accessory proteins such as ALIX, VPS4 and VTA-1[45,47]. Briefly, ubiquitinated proteins on the endosome surface are recognized by Hrs (a component of ESCRT-0 complex)[48,49] and drive the assembly of remaining members of ESCRT-0 via binding to phosphoinositide (PtdInsp3) on the endosomal membrane[50–52]. ESCRT-0 then recruits ESCRT-I[53,54], which recruits ESCRT-II to drive the invagination of the endosomal membrane[55–57]. This is followed by dynamic recruitment of the ESCRT-III components, which promotes the budding of vesicles by facilitating membrane scission around the stalk of ILVs with the help of VPS4 [58–62]. Apart from the canonical ESCRT pathway for ILV biogenesis, multiple non-canonical pathways have been identified where accessory component ALIX plays a critical role. These non-canonical pathways induce ILV biogenesis independent of ubiquitinated proteins and ESCRT-0, but utilize ESCRT-III and VPS4 for membrane scission[45]. To facilitate this, ALIX binds either to syndecan-syntenin complexes[63,64] or to lysobiophosphatic acid (LBPA)[65,66] or protease-activated receptor[67] on the endosomal membrane and induces ILV budding by recruiting ESCRT-III and VPS4 utilizing Bro-1 domains in ALIX. Similarly, HD-PTP, another Bro-1 domain-containing protein, drives ILV biogenesis by recruiting ESCRT-0, ESCRT-I and ESCRT-III[68,69].

Importantly, MVB formation is not solely dependent on ESCRT assembly, as evidenced by a study where simultaneous knockdown of ESCRT complexes did not abolish the MVB biogenesis in cells[70], highlighting the existence of ESCRT independent pathways. One such pathway is dependent on sphingomyelinase, where the formation of ceramide in the endosomal membrane can trigger ILV formation[71,72]. Furthermore, by clustering into tetraspanin-enriched microdomains, CD63, an exosomal specific tetraspanin, can trigger ILV formation[73–75]. The existence of multiple parallel pathways illustrates how heterogeneity within the exosome population may arise.

Although MVBs are primarily targeted for degradation in lysosomes after their formation, regulatory mechanisms exist that direct the MVBs to the plasma membrane for subsequent exosome release. For instance, diseases involving a defect in the lysosomal function use secretory pathways for efficient clearance of defective proteins and lipid recycling[76,77].

Similarly, disrupting lysosomal function by inhibiting the endosomal proton pump V-ATPase, which maintains acidic pH in lysosomes, leads to enhanced exosome release[78,79]. The balance between degradation and secretion can be narrowed down to the pathway used for MVB formation. For instance, MVBs originating from clustering of ubiquitinylated proteins[57,80,81] or via the LBPA-ALIX pathway are targeted towards degradation[45,82], whereases ubiquitin-independent pathways such as ALIX-syntenin-syndecan are destined for exosome release[63,83]. Importantly, different cell types may have other preferences towards the choice of the pathway it employs for MVB biogenesis and coordinate exosome secretion by regulating the fate of MVBs.

1.3.1.2 Transport of MVBs

The intracellular transport of MVBs to the plasma membrane shares similarities with the transport of other vesicles in the cell. MVB transport involves molecular motors like dynein or myosin, which shuttle them unidirectionally along the actin and microtubules cytoskeletons of the cells with the help of Rab GTPases. In support of this, polarised exosome secretion in immunological synapses between T cells and antigen-presenting cells is driven by the transportation of MVBs along the microtubule network[84]. In addition, the cytoskeletal regulatory protein cortactin promotes exosome release in cells by stabilizing cortical actin-rich docking sites[85,86]. Apart from cytoskeletal proteins, the transport of MVBs is tightly regulated by Rab GTPase molecular switches. For instance, Rab 27A and Rab 27B have been shown to be essential in the secretion of exosomes in various tumour cell types[87–90]. Importantly, Rab 27 is not expressed in all cell types, therefore involvement of Rab GTPases in MVB transport is cell type specific. This has been shown for oligodendroglia cells and hematopoietic K562 cells where exosome secretion is dependent on Rab35 and Rab11 respectively[91 92].

1.3.1.3 Fusion of MVBs with the plasma membrane

The final step for the release of exosomes is the fusion of MVBs with the plasma membrane. The membrane fusion is a highly regulated process in the cell and is primarily driven by the soluble NSF attachment protein receptor (SNARE) family of proteins. The family is comprised of more than 60 members in total and constitutes the major components of the cell membrane fusion machinery[93]. For the release of MVBs, vesicle-associated SNARE protein, i.e., VAMP (also known as v-SNARE), interacts with the target membrane protein syntaxin and SNAP (also known as t-SNARE). Interaction of VAMP and syntaxin along with the SNAP25/SNAP23 forms a trans SNARE complex which drives membrane fusion followed by pore formation[94]. Similar to Rab GTPases, there are range of v-SNARE and t-SNARE, and the expression of these pairs are cell type specific. For instance, VAMP7 has been shown to regulate EV release in K562 cells[95] but not in MDCK cells[96].

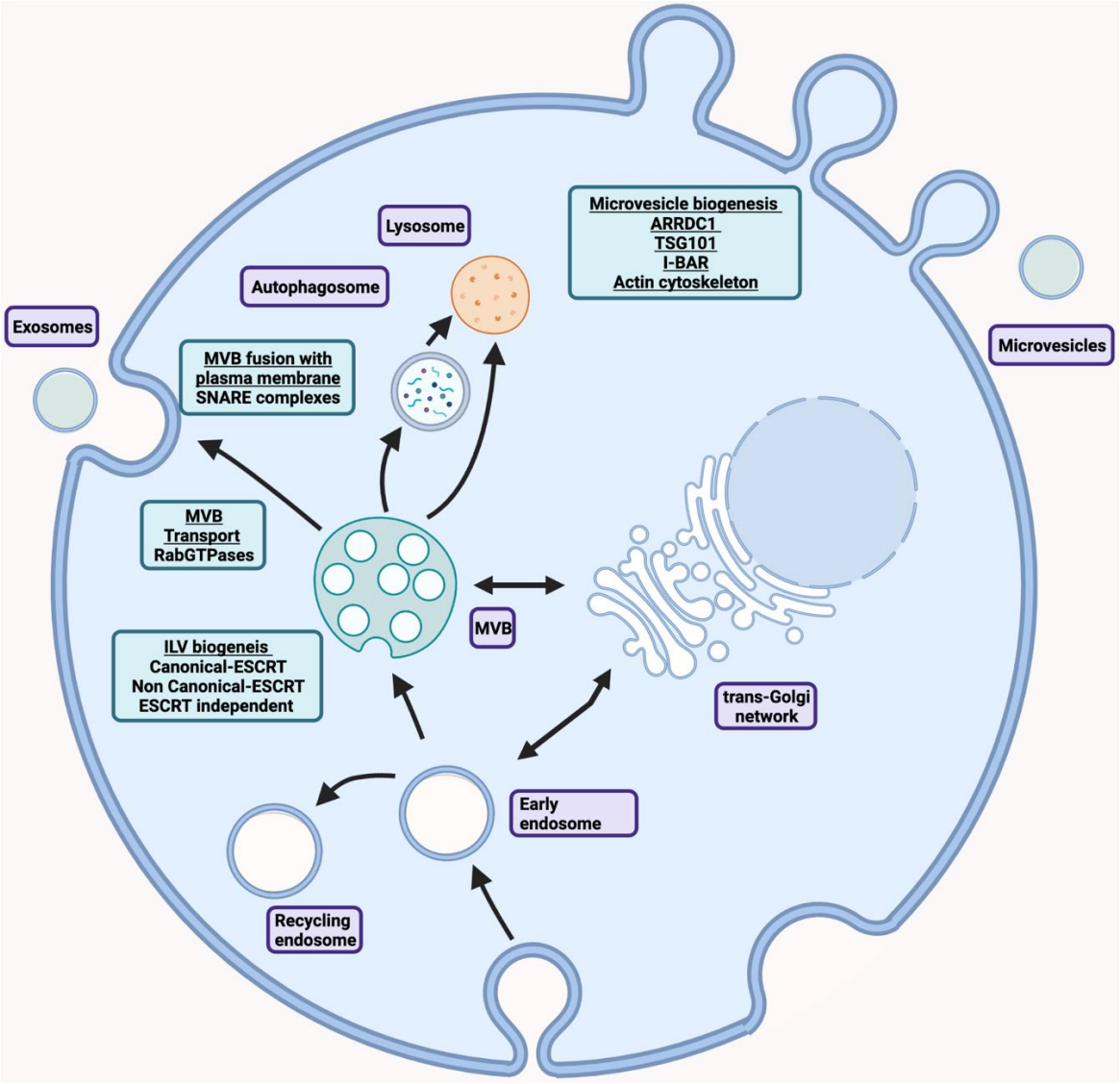

Figure 3. Illustration of EV biogenesis. Brief description of biogenesis pathways of exosomes and MVs.

1.3.2 MV biogenesis

Several independent molecular mechanisms of MV biogenesis have been identified in recent years. The process of its formation has been shown to arise from budding, shedding, pearling or scission of the plasma membrane[34]. Some of these mechanisms are directly related to lipid arrangements at the plasma membrane, changes in membrane peripheral protein composition, and Ca^{2+} levels[2]. For example, one mechanism involves calcium-dependent lipid rearrangement enzymes such as floppases, flippases and scramblases, which causes shuffling of the phospholipids to drive membrane bending along with cytoskeleton rearrangements, followed by budding of MVs from the plasma membrane[97,98]. Another mechanism involves direct recruitment of TSG101 and VPS4 by ARRDC1 at the plasma membrane to induce budding and scission of MVs[43,44].

Notably, the plasma membrane is a highly versatile fluidic structure of the cell, having multiple actin-based protrusions and extensions that facilitate sensing of the microenvironment. Recent evidence suggests that these plasma membrane structures, such as filopodia, microvilli, and retraction fibres, serve as a hotspot for MV biogenesis. The release of MVs through plasma membrane structures has been shown to be regulated by a number of proteins. The majority of these are involved in the regulation of membrane protrusions. For instance, inhibiting the actin polymerisation dramatically increased MV production in osteoblast cells[99]. A similar role of actin in inducing MV biogenesis was also observed in megakaryocytes[100] and chondrocytes[101].

Similarly, BAR proteins that are involved in inducing membrane vesiculation were shown to drive the formation of MVs by inducing filopodia scission [102]. Furthermore, overexpression of HSA3, which is involved in the generation of unbranched glycosaminoglycan hyaluronic acid, markedly increased the filopodia structure and MV release from the cells[103]. Similar to exosomes, multiple pathways have been described for MV biogenesis, although the possibility of the existence of all biogenesis pathways simultaneously in a cell is implausible.

1.4 PURIFICATION OF EV

Purification of EVs is a significant challenge in the research field as the extracellular environment, both in biofluids and cell culture supernatants, are rich in protein aggregates, lipoprotein complexes, non-EV bound RNA, cell debris and a heterogeneous pool of EVs[104–107]. Each of these components can have a physiological effect on the recipient cell[108]. Therefore, it is critical to have a robust EV purification method in place that enriches EVs to dissect their biological-, therapeutic-, and diagnostic role. Based on the desired EV application, different purification methods may need to be combined to achieve a highly pure EV preparation[109–111]. For example, EV purification from plasma is challenging as it is rich in albumin, lipoproteins and protein aggregates and these contaminants overlap with EVs in various physiochemical parameters [112–114]. Hence, the purification of EVs is of great importance in all areas of EV research. Since the first use of high-speed centrifugation for purifying EVs, the purification methods have evolved rapidly with the help of expanding knowledge about EVs[114] and now can be isolated based on various physicochemical properties such as density, size, charge, or affinity to specific biomolecules on the surface of EVs.

1.4.1 Ultracentrifugation (UC)

Centrifugation-based EV purification methods are still most widely used across the EV field[115]. The traditional process involves a series of low-speed spins to clear cell culture supernatants or biological fluids from cells, apoptotic cell debris, and other large microscopic particles. The precleared supernatant is further processed using multiple high-speed centrifugations ranging from 10,000g to 200,000g to recover large vesicles to small vesicles, including exomeres[30,116]. Since the separation of EVs is based on size and density, protein

aggregates tend to copurify with EVs[117]. For enhanced EV purity, density gradient centrifugations with 30–60% sucrose cushion or iodixanol can be employed to the remove vesicle-free proteins or protein-RNA aggregates[118,119]. Although UC on a density gradient yields pure EV populations, the method itself is highly variable due to user dependency. Furthermore, particle disruption, aggregation, and lack of scalability are still significant limitations of the ultracentrifugation-based purification methods[120,121].

An alternative method employing low-speed centrifugation for EV isolation is through precipitation either by addition of polyethylene glycol (PEG), or organic solvent (Protein Organic Solvent Precipitation technique)[122,123]. However, these approaches aim at precipitating both vesicular and non-vesicular proteins in the sample. Therefore, they are not considered as EV purification methods, and their use should be avoided.

1.4.2 Size exclusion chromatography (SEC)

SEC is a widely used chromatography method that is used across various research fields. SEC serves as a mean to separate molecules based on molecular size. Briefly, the sample which acts as a mobile phase is passed through a porous stationary phase[124]. Smaller particles will be able to traverse through many pores as compared to bigger particles, hence resulting in differential elution profiles where bigger particles will take a shorter path and elute first, followed by smaller vesicles and then non-exosomal proteins[121]. Pore size and density of the stationary phase is based on the polymer used and can be modulated by selecting from the number of gel polymers available such as crosslinked dextrans, agarose and allyldextran[125]. Due to the limitation of mobile phase volume, which can be subjected to SEC, a pre-processing step such as ultrafiltration to concentrate the sample is often performed[121,126,127]. Usually, precleared cell culture supernatants are subjected to ultrafiltration devices either with dead-end or tangential flow filtration systems with a molecular weight cut-off ranging from 10 to 1000 kDa. Dead-end filtration is more suitable for small-scale applications while tangential flow filtration is more appropriate for large-scale production [128,129]. Furthermore, by using ultrafiltration with higher molecular weight cut-off values, relatively pure and intact vesicle preparations can be obtained, hence further improving the efficiency of the downstream purification system[130]. EVs purified by SEC have better integrity and purity as compared to UC- based approaches, as demonstrated by several studies[120,121]. In addition, the scalability of SEC makes it a promising candidate for EV purification for GMP production[131–133]. However, since the separation of EVs in SEC is based on size, the risk of co-elution of VLDL, chylomicrons, and LDL is relatively high in the case of EV purification from plasma[112]. Furthermore, due to the long processing time and lack of throughput, the applicability of SEC in EV based diagnostics is complicated.

1.4.3 Alternative methods for EV purification

Apart from using density and size, alternative methods have emerged which utilize molecular, biophysical or biochemical attributes of EVs to segregate EVs from other non-

vesicular contaminants and aim to delineate the heterogeneity of EVs. One way of efficiently capturing EVs in biological samples is to target specific surface markers known for a particular EVs population[114]. Up until now, a wide range of EV markers has been identified, which has led to the development of various immune affinity-based approaches for EV purification. The most commonly used immunoaffinity approaches are directed towards EV specific tetraspanins such as CD9, CD81 and CD63[134]. In addition, some other protein targets have also been used, such as MHC antigen[135] and heat shock proteins[136]. Apart from protein-based targets, targeting lipids such as phosphatidylserines by Annexin V[137] or targeting proteoglycans or other glycocalyx structures by heparin[138], and lectins [139–141] has been used for affinity-based EV purification.

Immunoaffinity based EV purification allows for efficient isolation of pure EVs from complex biological fluids in a high throughput manner, with minimum user dependency on the purity and yield, therefore it has been deemed ideal for EV based diagnostic applications[142,143]. However, one of the primary challenges with affinity purification is to elute EVs without affecting the integrity, although efforts are being made for non-destructive retrieval of EVs from the affinity columns but these methods lack scalability, which is critical for EV purification for therapeutic applications.

In addition to immunoaffinity, several other technologies have been developed or repurposed for EV isolation. Amongst others, these include ion-exchange chromatography,[146,147] asymmetric flow field fractionation[148,149] and microfluidic-based systems[150,151].

1.5 EV CHARACTERIZATION

EVs are a heterogenous pool of vesicles that contain proteins, a variety of nucleic acid species ranging from small RNA to full-length mRNA and DNA, and lipids[152]. Since EVs are smaller than the wavelength of visible light, reliable detection of EVs is a challenging task. However, over the years, various methodologies have been developed to achieve reliable quantification of EVs[153].

Levels of any of the biomolecular cargo or the vesicle as a structure can serve as a basis for EV quantification. Based on this, various means of quantifying EVs have been developed. Technologies which measure hydrodynamic sizes, such as nanoparticle tracking analysis (NTA)[154] and resistive pulse sensing[155], are sensitive and provide a robust means for sizing and determining EV concentrations. However, these methods fail to distinguish an EV from other similar sized non vesicular particles, hence the specificity of the assay is entirely dependent on the choice of purification method as certain methods tend to co purify lipoprotein complexes and protein aggregates [117,156,157].

Recently, flow cytometry-based applications have emerged to quantify EVs at a single vesicle level[158–162]. However, considering the size of an EV, especially the small EVs, the amount of light scattered fails to trigger the sensor on conventional flow cytometers.

Therefore, a flow-based application needs to be coupled with detection of a fluorescent antibody or a fluorescent lipophilic dye to get a robust signal[158–162]. Apart from light scattering based tools, transmission electron microscopy (TEM) can be used for the quantification of the EVs, but size of a vesicle can differ largely due to the process of sample fixation, which could lead to swelling or shrinking of the EVs[163].

EVs can also be quantified by measuring the cargo loaded inside the EVs. In this regard, total protein amount, total lipid content or total RNA have been used as a way of determining EV amounts[25,164]. Especially total protein content is still widely used across the research field[165]. Although these methods are high throughput and do not require expensive machinery, the propensity of measuring non-vesicular contaminants in EV preparations is high. Therefore, measuring the levels of EV-associated protein, for instance, CD63 or CD81, can provide reliable means of quantification but may not reflect the heterogeneity of the EVs[166–171].

Overall, despite the technological advancement and availability of a range of highly sensitive methods, accuracy in EV analytics is still a challenging goal that needs to be achieved. In addition, particles/ml is an arbitrary unit as one sample measured on different equipment can yield different values.

1.6 EV COMPOSITION

Despite limitations associated with EV purification and characterization, the content of EVs is heavily investigated. With the easy availability of multi-omics approaches, the diversity of EV associated proteins, nucleic acids, lipids and metabolites are being unravelled (Figure 4).

Notably, most of the EV characterisation studies have performed analysis on the bulk EV population, which may not reflect the heterogeneity of the EVs. Nevertheless, with the innovation in purification technology, future studies may delineate the biomolecular cargo in different EV subtypes. This section will summarise the findings on the three most enriched cargos in EVs.

1.6.1 The protein content of the EVs

EVs carry a broad range of transmembrane proteins, membrane associated proteins and luminally loaded soluble proteins. Various studies have performed proteomic characterization of EVs from multiple cell lines and tissue explants. For instance, Hurwitz *et al.* performed proteomic characterization of EVs derived from 60 different cancer cell lines and identified 6071 proteins in total, out of which 213 proteins were common to all cell types, and only a minority were exclusive for a specific cell source[172]. Similarly, Kugeratski *et al.* identified a total of 3759 proteins in EVs derived from 14 cells lines, out of which 642 proteins in total were unique for different cell types [173]. Furthermore, Hoshino *et al.* analysed 497 EV preparations from cell lines, tissue explants and plasma from both mouse and human and identified some common homology in the protein signature of the EVs[174]. These studies

at large reflect the fact that the majority of the EV proteome is ubiquitous and is enriched in proteins involved in the biogenesis and their interacting partners. In addition, only a minority of the proteome reflects the cell-type specificity. Importantly, the mechanism involved in sorting or loading of cell type-specific proteins is yet to be determined but a majority of these proteins are cell surface receptors which could indicate that they originate from plasma membrane shedding.

EVs are highly enriched in various tetraspanins such as CD9, CD37 CD63, CD81 and CD82[175,176]. Tetraspanin family proteins are not enzyme-linked receptors nor catalytic receptors but they may promote the sorting of protein cargos, especially tetraspanin interacting proteins such as Integrins[177], ICAM-1[178], IgSF-8[179], MHC class II proteins[12,176] and syndecan[63]. However, various reports and some unpublished work from our group failed to see any differences in EV numbers or EV proteome upon overexpression or silencing of either CD63, CD81 or CD9[180,181]. These discrepancies could be due to differences in cell types used as different cells have different biogenesis pathways and tetraspanin mediated sorting of cargo could be linked to a separate MVB biogenesis pathway. Furthermore, based on their cellular localization, it has been speculated that tetraspanins such as CD63 are exclusively present on EVs of MVB origin, whereas CD81, which is primarily localized on the cell surface are preferentially sorted into MVs, originating from the plasma membrane[29,41,182]. This is clearly reflected by the fact that CD63 positive vesicles are CD81 low or negative and vice versa[41]. In addition to tetraspanins, there are also other scaffolding transmembrane proteins that are associated with EVs, such as Flotillin 1 and Flotillin 2[183]. Other studies have also identified receptors such as IL-6R[184], EGFR[185], T cell receptor[186], chimeric antigen receptor[187], Notch receptors[188] and GPCR receptors[42,189] on the surface of the EVs. Furthermore, the surface of EVs is also rich in ligands and proteases such as PD-L1[190], TGFB[191], and ADAM proteases[192]. Apart from transmembrane proteins, the surface is also rich in membrane interacting proteins, specifically proteins with GPI anchors, for example, complement inhibiting proteins DAF and MAC-IP[193]; cell surface proteoglycan glypican-1[194]. Additionally, on the inner leaflet, a range of proteins has been identified, such as small GTPases, which are involved in the biogenesis and adhere to the inner leaflet by prenylation PTM[91,195,196]. In addition to prenylated proteins, myristoylated proteins such as BASP-1[197] and Src signalling kinases[195] also interact with the inner leaflet and sort into EVs. Similarly, HIV Gag also uses N-terminal myristoylation for loading into viruses or EVs[39]. Other PTM modifications which have been shown to drive cargo sorting are ubiquitination, SUMOylation[198,199] and phosphorylation[200].

Other proteins, which are also found to be abundant in EVs, interact with- or are part of ESCRT complexes such as ALIX, TSG101 and syntenin [57]. Apart from biogenesis related proteins, EVs are also enriched with molecular chaperones such as Hsp70, Hsp90 and Hsp20 [201–203]. Finally, cytosolic proteins such as actin and tubulin are also sorted into EVs and are most likely being sorted upon MV shedding from the plasma membrane[172].

1.6.2 RNA sorting into EVs

The extracellular environment is rich in a variety of nucleic acids, either encapsulated in the EVs or bound to protein or lipoprotein complexes. However, the unique ability of EVs among this diverse extracellular landscape to functionally deliver nucleic acid to different cell types has led to an exponential increase in effort to characterise EV-associated nucleic acid cargo and its implication in disease pathophysiology[14–16]. A range of nucleic acids has been identified in EVs, including small RNAs, mRNA, circular RNA, and even dsDNA[204–206]. However, existence of dsDNA in EVs is debatable as DNA is considered a contaminant co-purifying with EVs and it was recently shown to be secreted via an autophagy-dependent mechanism in non-vesicular particles [29]. Furthermore, in our hand upon treating the EVs with DNases the majority of the DNA is lost, indicating that the majority of the DNA is outside of the EVs, and co-purification could be due to membrane association.

In terms of RNA, EVs are primarily enriched in tRNAs, miRNAs, snRNAs, Y RNAs, snoRNAs, fragmented rRNAs, vault RNAs, full length and fragmented mRNA[105,207,208]. However, the exact composition is still a debatable topic as different studies have reported different compositions of small RNAs in EVs[209]. This is due to the fact that the majority of the extracellular RNA is non-vesicular, and the choice of purification method can heavily influence the observed RNA content of the EVs[105,156,210]. In addition, due to the high degree of sequence complementarity of small RNAs across different species, it is hard to discriminate the small RNAs derived from FBS used for culture conditions from their human counterparts[211]. Therefore, some of the highly enriched miRNAs in EVs reported previously could be due to FBS contamination[212,213]. Similarly, serum-free media supplements were also shown to be enriched with miRNA 451a and 122-5p[214].

Even after removing common contaminants, the RNA content is highly diverse in EVs. The molecular mechanisms behind RNA sorting into EVs have just started to unravel. Based on the literature on EV associated RNA, sorting of RNA cargo can be mediated by two mechanisms; either by active loading mechanism or by passive loading[215]. Passive loading into EVs is primarily dictated by the intracellular concentration of specific RNAs, and its enrichment is entirely cell-dependent and one of the critical factors for diagnostic applications[216,217]. Apart from passive loading of RNA cargo, active loading mechanism also exist as implied by certain trends in the nucleic acid cargo. For instance, 3'UTR enriched mRNA fragments are enriched into EVs[207,208,218]. Similarly, miRNA with 5' oligopyrimidine sequences are enriched in EVs[219]. Apart from this, some RNAs are sorted into EVs exclusively in a sequence specific manner by interacting with RNA binding proteins. For instance, GGAG motif carrying miRNAs are sorted into EVs by hnRNPA2B1[199]. Furthermore, hnRNPU has been shown to regulate the sorting of miRNA 30c-5p into large EVs[220]. The SYNCRIP protein mediates sorting of various miRNA into EVs by binding directly to common extra seed sequence called hEXO motif [221,222]. The YBX1 protein has also been shown to mediate EV sorting of miRNA and small ncRNA[208,223]. In addition to

this, LC3, an important autophagy component, mediates the loading of multiple RNA binding proteins such as HNRNPK and SAFB and small non-coding RNAs into EVs[224]. Apart from this, Dicer independent miRNA-451 that requires Ago2 catalysis was found to be selectively enriched in EVs and could be used for loading therapeutic shRNA into EVs[225]. Interestingly, the majority of RNA binding proteins carry arginine-rich or glycine-rich motifs and tend to oligomerize under stress conditions[226,227]. Therefore, it could be hypothesized that oligomerization of these proteins mediates protein-RNA loading into EVs similar to Gag proteins into EVs or retroviruses[39]. These findings correlate well with previous findings indicating that most of the extracellular RNA is outside of vesicles[29,105,228]. Supporting this fact, a recent study identified another Extracellular nanoparticle called "Supermeres" which are 25-35 nm in diameter and carry up to 60% of the total extracellular RNA, whereas, only 25% of total extracellular RNA was identified in small EVs[228]. These studies reflect the fact that the RNA biology of EVs is still vague, and the purification method is critical in order to uncover the actual RNA content of EVs.

1.6.3 Lipid content of the EVs

The membrane of EVs is enriched with a variety of lipids, but very few studies have performed quantitative lipidomic on the EVs. Their membranes are highly rich in cholesterol, sphingolipids, phosphatidylserine (PS) , phosphatidylinositols (PIs), phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), ceramides and glycosphingolipids [229–233].

In terms of distribution, lipids are distributed in an asymmetric fashion on the plasma membrane of cells, with sphingolipids and PC being present on the outer leaflet and other phospholipids such as PS and PE on the inner leaflet[234]. However, in EVs membrane PE and PS are enriched on the outer leaflet[235,236], a similar distribution of lipids is also present on apoptotic bodies, where exposed PS work as a "eat me" signal to facilitate clearance by phagocytes[237]. However, Lai *et al.* demonstrated that EVs positive for Annexin V, which recognises exposed PS membrane, were relatively empty in exosomal markers such as TSG101, ALIX and tetraspanins[170]. Similar results were also reported by Matsmura *et al.*, where PS exposed EVs had lower density then other EVs[238].

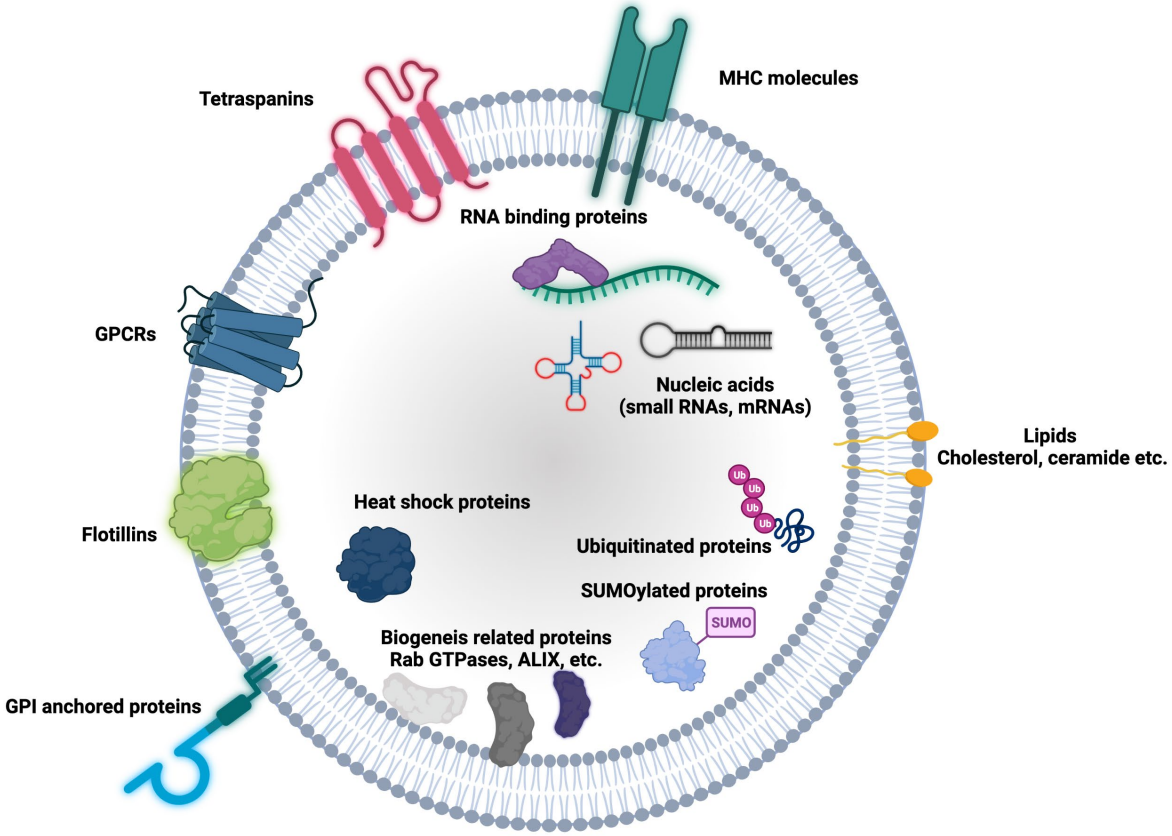

Figure 4. A schematic representation of EV associated cargos.

1.7 EV IN VIVO AND IN VITRO UPTAKE

EVs have been shown to be essential for cell-to-cell communication and can transfer large macromolecules such as mRNA and proteins to recipient cells. Due to their relatively small size, understanding there *in vitro* and *in vivo* behaviour and role is rather challenging. Therefore, various EV labelling tools which allow for exclusive imaging of EVs in a complex extracellular environment are being developed to dissect the spatiotemporal properties of EVs.

1.7.1 Tools for imaging EVs

Briefly, EV labelling can be achieved in two ways, either by general labelling of EV-associated macromolecules or by labelling a specific macromolecule in an EV. There is a range of tools available for labelling EVs with a tracer, both endogenous and exogenous strategies (Figure 5). This section will discuss a few of the most commonly used EV labelling strategies.

1.7.1.1 Lipid dyes

Lipophilic tracer dyes have been widely used for EV labelling[239,240]. The dyes usually consist of a fluorophore conjugated to a lipophilic functional group which facilitates the insertion of

the tracer into the lipid bilayer by non-covalently interacting with EV lipids. Based on this, a number of dyes (for example, PKH67, DiR/DiL/DiD) are available that cover a broad range of the emission wavelengths such as infrared spectrum which is advantageous for *in vivo* applications due to better tissue penetration [239–241]. Moreover, these dyes allow for quick and efficient labelling of EVs without the need to alter the producer cells. Although being convenient to use and permitting labelling all EVs, these dyes tend to aggregate or form micelles and can potentially label non-EV particles[242]. Furthermore, there is a considerable risk of transfer of EV bound dye to the plasma membrane of cells as the interaction is non-covalent. In addition to these limitations, labelling with lipophilic tracer dye has shown to alter the characteristics of EVs[243]. A similar observation was made by us where labelling of EVs with DiR influenced the biodistribution of EVs *in vivo* [244]. Apart from lipid anchors, EVs can be labelled with fluorophores by covalent reaction of fluorophore NHS ester with the amine group of EV surface proteins[245,246]. However, these covalent conjugation strategies can potentially alter the EV surface proteome, which may affect their interactions with other proteins. In addition, this approach lacks specificity and may label non-vesicular proteins. Notably, the current generation of dyes and tracers are highly stable and have a half-life of a few days to weeks and do likely not reflect the natural half-life of an EV[247,248].

1.7.1.2 Radiotracers

Apart from fluorescent dyes, EVs can be labelled with PET tracers (e.g. 99mTc-HMPAO[249], 125I-IBB[250], 111-Indium-oxine[251]) either by conjugation either to lipophilic groups or to amine groups on the EV surface. MRI has also been used for imaging the biodistribution of EVs. Super magnetic iron oxide nanoparticles can be loaded into EVs either by exogenous loading through electroporation or endogenous loading by feeding cells with 5 nm SPIONs[252,253]. The advantage of radiolabelling and MRI is the exceptional sensitivity *in vivo* over other light-based reporters, but half-lives of these tracers may not truly reflect EVs half-life in tissues. Furthermore, these modalities are expensive and lack throughput.

1.7.1.3 Fluorescent and bioluminescent proteins

In addition to a variety of exogenous labelling strategies, EVs can be genetically engineered with fluorescent or bioluminescent proteins to label all the EVs or a specific population thereof[181,244,246,250,254,255]. For labelling a specific population, producer cells are genetically engineered to express a reporter protein fused to an EV sorting domain to dictate the loading of the reporter protein into EVs during the biogenesis. For example, a fusion protein of CD63 and EGFP can drive the sorting of GFP into EVs and label 30-40% of the EVs, each carrying 30-60 EGFP molecules on average[181]. This approach is not limited to CD63 since other EV sorting domains can also be exploited for labelling EVs (e.g. CD9, CD81, syntenin, Gag) [181,254]. Although it seems straightforward, the EV engineering efficiency with certain EV sorting domains such as ALIX, SIMPLE and syndecan is relatively low [181]. This could be due to potential loss of the protein's function due to fusion of a reporter protein. Overall, genetic engineering approaches provide an efficient way of labelling a specific EV population

either with fluorescent proteins (GFP, RFP, etc.) or bioluminescent proteins (Gaussia-, Firefly- and Nano-luciferase). However, these approaches fail to label all EVs and require genetic engineering of the producer cells, which could be challenging for some cell sources. In addition, overexpression of certain EV sorting domains may alter the EV biogenesis pathway or EV proteome.

An ideal EV reporter or labelling strategy does not exist at this moment. With the multitude of EV labelling strategies available, each has some degree of advantage and disadvantage; therefore, it is essential to select a method based on the feasibility.

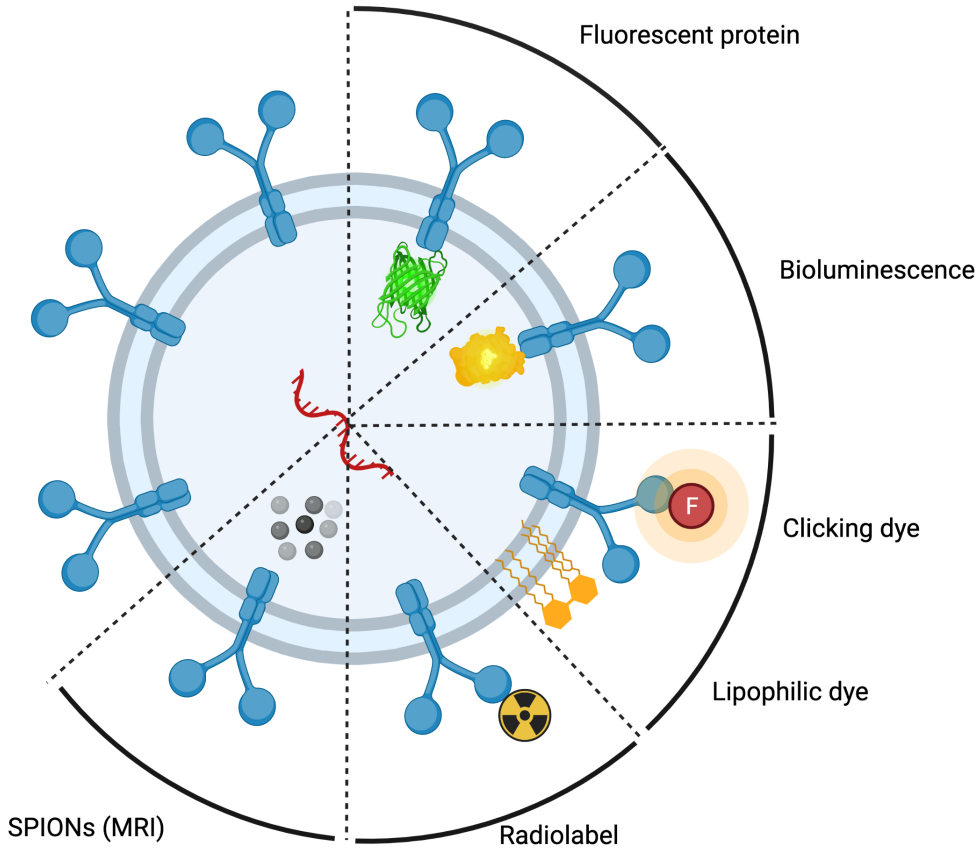

Figure 5. Exogenous and endogenous strategies for labelling EVs for *in vivo* and *in vitro* applications.

1.7.2 *in vitro* uptake of EVs

Since the first report of RNA transfer between cells through EVs, much of the focus has been on understanding EV uptake pathways. In an elegant study by Heusermann *et al.*, EVs were observed to surf individually on filopodia before being endocytosed, sharing striking similarities with the *in vitro* uptake profile of enveloped viruses[256]. Similar to viruses, several energy-dependent pathways such as micropinocytosis, clathrin-dependent, caveolin-

dependent or lipid raft mediated endocytosis processes have been reported to be involved in uptake of EVs into recipient cells[257–259]. Due to the existence of a heterogenous EV population and a variable surface protein composition, the involvement of multiple uptake pathways is expected. The presence of lectins, tetraspanins, integrins and proteoglycans on the EV surface has shown to be the major driver of EV uptake through energy-dependent endocytosis [260–264]. In contrast, evidence of direct fusion with the cell membrane is limited[265]

Importantly, evidence on how the luminal cargo of EVs escapes the endocytic vesicle is still largely lacking, with the possibility of either a full fusion between EV and endosomal membrane or complete cargo degradation in lysosomes. This is a critical aspect on which contradictory research exists. As some recent reports have observed no to little cytosolic delivery of cargo with EVs, and the majority of the EVs is destined for lysosomal degradation upon being taken up[266–268]. On the contrary, some studies have shown that EVs have an inherent ability to escape from endosomes[269–272]. There are various factors that could be driving this discrepancy. The difference in cell source could be a potential player as some early developmental cells express ERV ENV genes such as syncytin-1 and syncytin-2, which could potentially induce endosomal escape by fusion[273–276]. Furthermore, recent evidence suggests that a small percentage of ILVs which are secreted as exosomes tend to retro fuse with the MVB membrane during biogenesis, indicating a possibility that a small proportion of exosomes may be able to induce membrane fusion[277].

1.7.3 *in vivo* uptake of EVs

Since the first study showing CNS delivery of synthetic siRNA by EVs, interest in EVs to use as drug delivery technology has gained increasing attention[278]. For translational research it is of uttermost importance to understand the distribution of EVs *in vivo*. Numerous studies in the past decade have showcased EVs *in vivo* biodistribution[239,244,246,279]. Since the early use of lipophilic dyes, EV imaging modalities have evolved, and with the development of endogenous labelling strategies, biodistribution can be evaluated at much higher sensitivity. The majority of studies have shown that EVs tend to primarily accumulate in the liver, lungs, and spleen and to a lesser extent to other organs and tissues such as the brain, muscle, heart, and kidneys[280]. This pattern is quite reflective of the endothelial architecture as organs with discontinuous endothelium, such as the liver and spleen, having the highest accumulation[281] while organs with smaller fenestrated endothelium have a lower accumulation of EVs and may require transcytosis in order to reach the organ, as shown for the CNS accumulation of the EVs[282]. Similar trend is also observed for therapeutic AAVs with CNS tropism (AAV9), where majority of the dose is sequestered in liver and only a small fraction targets CNS. Nevertheless, as discussed earlier, labelling EVs with bioluminescent or fluorescent proteins may reflect the true fate of EV cargo *in vivo*. Importantly, right after the uptake of EVs in different tissues, signals associated with luciferase protein tend to degrade over time despite these enzymes being highly stable, suggesting degradation of the protein cargo in the lysosomal compartment.

Furthermore, various studies have shown that the reticuloendothelial system (RES) is primarily responsible for the clearance of EVs similar to other nanoparticle- based drug delivery vectors, as EVs injected in animals with impaired innate immune and complement system had much slower plasma clearance and liver accumulation[251]. These results are also supported by various studies where either blockade of scavenger receptors on macrophages or sequestration of PS on the surface of EVs prevented rapid clearance and liver uptake[236,283,284]. Notably, a recent study investigated the protein corona on the surface of EVs in plasma and identified apolipoprotein and complement association with the EVs[285]. The EV protein corona had a high overlap with viruses and synthetic nanoparticles[285,286]. Since viruses and EVs share similar biogenesis pathways, it is not surprising that they share similarities in their uptake mechanism both *in vitro* and *in vivo*. This clearly implies that EVs are opsonised by complement proteins and are taken up by monocytes and macrophages in the liver (Kupffer cells) which forms the part of RES system. To circumvent the issue of clearance by RES, various engineering strategies have been employed on the surface of EVs. For instance, EV expressed CD47, a classical "don't eat me signal", prevented the uptake of EVs in circulating monocytes *in vivo* and extended EV half-life by 3-fold[287]. Similar observations were made in other studies where the expression of CD47 prevented macrophage clearance[288,289]. Furthermore, the expression of other CD47-like molecules such as CD55 and CD59 on EVs surface prevented complement activation *in vitro*[290].

Apart from understanding exogenous EVs *in vivo* biodistribution. Similar efforts have also been made for understanding the fate of endogenous EVs *in vivo*, although there are very few studies due to technical limitations to study them but the evidence suggests that the majority of endogenous EVs are also cleared rapidly either by endothelial cells or macrophages[255,291]. Importantly, all these studies have addressed biodistribution of the EVs in a specific tissue which may not reflect the bioavailability. Furthermore, it is highly likely that a specific population of EVs or a specific cell source derived EVs are bioactive[292]. Therefore, much of the focus should now be on addressing the bioactivity of EVs both for endogenous and exogenous EVs.

1.8 THERAPEUTIC APPLICATION OF EVS

The attribute of achieving body-wide distribution makes EVs a promising candidate for drug delivery, especially during an era where we have developed a range of novel biotherapeutics such as genome editing mega-nucleases, modified mRNA and other nucleic acid-based therapeutics, for which efficient *in vivo* delivery is still a significant milestone[293,294]. Apart from unique biodistribution capabilities, exogenous EVs have shown to be non-immunogenic and induce no toxicity in animals, and no adverse side effects have been reported in any of the clinical trials utilizing EVs as a therapeutic intervention[295–297]. However, a few studies have shown that EVs displaying tissues factors can induce blood coagulation [298,299] and that tumour cell-derived EVs contain hTERT mRNA which can transform non-malignant cells

into telomerase positive cells [300]. However, this is entirely dependent on the producer cells, as most of the cellular content is reflected in the EVs.

1.8.1 Innate therapeutic potential of EVs

MSCs innate immunomodulatory effects have been used to treat various inflammatory diseases in humans as a cell therapy. However, due to very few cells grafting in the body post treatment, the therapeutic effect of MSCs has been attributed to paracrine factors secreted by them such as soluble proteins and EVs[301]. Growing evidence suggests that EVs purified from MSCs can deliver immunomodulatory cargo for tissue regeneration and immunomodulation[301]. Furthermore, repeated administration of MSC derived EVs in a graft versus host disease patient showed therapeutic benefits without any sign of side effects[302]. Since this study, various biomedical applications of native EVs derived from various cell sources have emerged for a range of diseases. For instance, MSC-derived EVs have shown therapeutic response in animal model of traumatic brain injury[147], myocardial infarction[303], multiple sclerosis[304], acute lung injury[305], colitis[306] and GvHD[307]. Apart from inflammatory diseases, native EVs have shown great therapeutic promise for anti-tumour treatment, EVs purified from DC cells pulsed with tumour antigen can be utilized for cancer immunotherapy applications and previously have been under clinical investigation for the same[308]. In addition, NK cell-derived EVs exhibit cytolytic activity against tumour cells both *in vitro* and *in vivo*[309].

1.8.2 Engineered EVs for drug delivery

The use of EVs is not only limited to its native form, since the intrinsic ability of intracellular communication by EVs can be tailored for the delivery of biotherapeutics. Since the first demonstration of using engineered EVs for CNS delivery of synthetic siRNA[278], numerous sophisticated technologies have been developed to load variety of biotherapeutic cargos into EVs and decorate the surface with targeting moieties. There are two ways by which EVs can be engineered (Figure 6). The below section will summarise some of the key technologies used for EV engineering and their applications.

1.8.2.1 Exogenous engineering

Exogenous loading into EVs usually involves the incorporation of cargo either on the surface or in the lumen of pre-isolated EVs by various physical or chemical methods, including co-incubation, sonication, and electroporation[310,311]. By this, EVs isolated from conditioned medium can be loaded with a variety of nucleic acid cargos ranging from siRNA and ASOs to larger nucleic acid drugs such as mRNA. The applicability of this technology was first showcased by Alvarez-Erviti *et al.*, where EVs engineered with brain targeting peptides and electroporated with siRNA could cross the blood-brain barrier and achieve silencing in the cerebral cortex of mice following intravenous administration[312,313]. However, various reports have shown that the majority of the cargo gets aggregated upon electroporation and co

sediments with EVs upon purification[314,315]. In addition, other physical methods such as sonication or extrusion, which have been used for protein loading into EVs, can potentially affect the integrity of EVs. Therefore, various alternate strategies have been developed for the exogenous loading of EVs. One such approach was recently described for loading nucleic acid drugs such as siRNA, where a cholesterol tag associated with siRNA facilitates surface decoration by interacting with the lipid bilayer of EVs[316,317].

Another strategy gaining attention is the generation of hybrid EVs, where mixing with lipid nanoparticles (LNPs) or cell-penetrating peptide (CPP) nanoparticles loaded with biotherapeutic cargo allows for the generation of EV-LNP or EV-CPP hybrids[318]. This strategy has been shown to be viable for loading siRNA, ASOs, mRNA and even CRISPR Cas9 RNP. Notably, most exogenous loading methods are focused on luminal loading of the drugs, and only limited methods are available for surface engineering of EVs. For instance, a recent study by Pham *et al.* utilized protein ligation enzymes sortase from bacteria for surface engineering of EVs with nanobodies or targeting peptides[319]. Similarly, different click chemistry methods have been utilized to functionalize the EV surface with targeting ligands[320]. Although these methods allow for efficient engineering, they can potentially alter the surface proteome or integrity of the EVs. Furthermore, exogenous loading of large proteins and big nucleic acid cargo is still inefficient. Therefore, alternative ways of loading biotherapeutics into EVs have been developed, which hijack the EV biogenesis pathways to load protein therapeutics.

1.8.2.2 Endogenous engineering

For endogenous loading, the parental cells are genetically modified by either pDNA transfection or through lentivirus mediated integration of transgene, to overexpress a desired therapeutic cargo either protein or RNA, with specific modifications to dictate active loading or without any modifications for passive loading into EVs during EV biogenesis. In the past decade, various endogenous engineering strategies have been described. One of the most used methods is to utilize tetraspanin proteins such as CD81, CD9 and CD63, which are highly enriched on the EVs surface[172]. Fusion of the desired protein of interest to either the N- or C- terminus of tetraspanins allows for efficient means of luminal loading into EVs[181]. In addition, some other EV sorting proteins such as syntenin, BASP-1, Syndecan-1 or MFGE8 have also been used for EV engineering[181,310,321]. Apart from loading therapeutic protein cargo, replacing the therapeutic protein with an RNA binding protein can drive the specific sorting of RNA, either mRNA or small RNA, into EVs[322]. Similarly, EV sorting domains with transmembrane domains such as Lamp2b or tetraspanins can be engineered to display targeting ligands or therapeutics on the surface of EVs. We recently described one such approach where EVs were surface engineered to display cytokine binding domains to sequester pro-inflammatory cytokines[323].

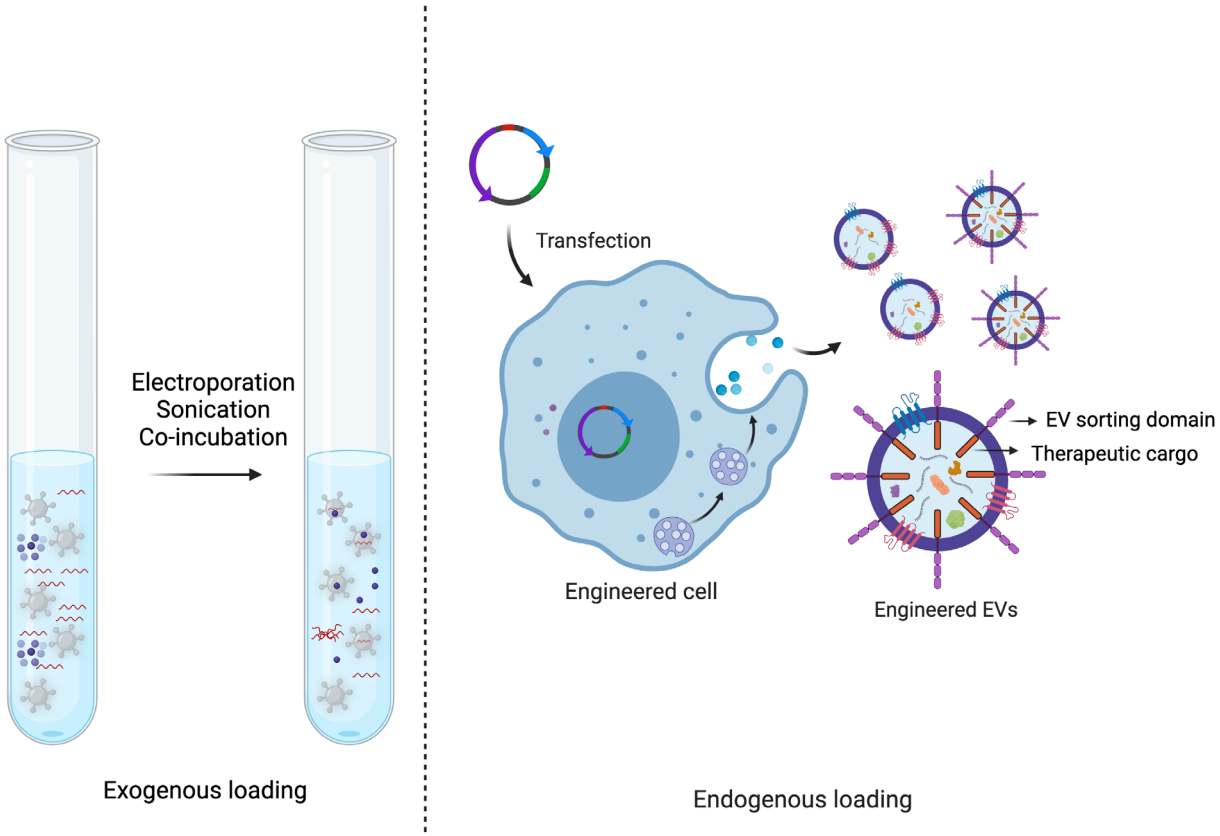

Figure 6. EV engineering and loading strategies.

1.8.3 Biomedical applications of Engineered EVs

By combining endogenous and exogenous engineering approaches, various therapeutic applications have emerged in the past decade. For instance, EVs engineered with CD47 and loaded with siRNA targeting mutated KRAS suppressed pancreatic cancer in multiple mouse models and improved overall survival[287]. Similarly, iRGD targeting peptides displaying EVs loaded with doxorubicin suppressed tumour growth *in vitro* and *in vivo*[324]. Furthermore, Wang *et al.* employed a totally novel approach for cancer immunotherapy[325]. Transfer of the nuclei of a tumour cell into an activated macrophage led to the generation of chimeric EVs, which homed into the lymph node and delivered cancer associated antigens to prime T cell activation. Apart from this, EVs have been endogenously loaded with mRNA encoding tumour suppressor genes such as PTEN for efficient, targeted delivery to glioblastoma *in vivo*[326].

The biomedical applications are not only limited to tumour cells. Engineered EVs efficiently delivered super repressor protein IkBa to alleviate symptoms and mortality in a septic shock mouse model[327]. In addition to this, RBC EVs have been used for the delivery of a variety of nucleic acid drugs such as antisense oligonucleotides, mRNA and miRNA mimics[328]. In terms of nucleic acid drug delivery, various other engineered EV platforms have shown remarkable therapeutic efficacy. For instance, Kojima *et al.* built an EXOtic device, which

enables efficient loading of therapeutic mRNA into EVs by endogenous loading and delivery to CNS [271]. Apart from large mRNA, Reshke *et al.* achieved shRNA loading by using a Dicer-independent and Ago2-dependent miRNA 451 scaffold and observed 10-fold more potent delivery of shRNA as compared to using state-of-the-art lipid nanoparticles[225]. Similar superiority of EVs over lipid-based carriers have been reported in other studies as well[295,329].

Overall, these developments have led to the initiation of various clinical trials using EVs as a therapeutic intervention, although none of these clinical studies was able to show efficacy based on the primary endpoint. This clearly highlights the fact that more in-depth knowledge about EV biology is needed to achieve a smooth translation from bench to bedside.

2 RESEARCH AIMS

Research contributions in the field of EVs over the past three decades have transformed the outlook of biology. The ability of EVs to transport bioactive molecules in several physiological and pathological processes has motivated many to use them as a drug delivery tool to deliver the undeliverable. Inspired by this, this thesis initially aimed to develop a sensitive tool for EV quantification *in vivo* and, lastly, to explore novel therapeutic applications of engineered EVs. The individual aims and objectives concerning the research articles are as follows:

2.1 PAPER I

- To develop an efficient bioluminescent-based EV subpopulation specific labelling strategy that allows for high throughput and low-cost EV quantification *in vitro* and *in vivo*.
- To evaluate various *in vitro* and *in vivo* applications of bioluminescently labelled EVs.
- To evaluate EVs *in vivo* pharmacokinetics and the effect of the route of administration on *in vivo* biodistribution.

2.2 PAPER II

- To develop a novel EV engineering platform for surface decoration of cytokine decoy receptors.
- To evaluate the therapeutic efficacy as an anti-cytokine drug *in vitro* and in three different *in vivo* inflammation models.

2.3 PAPER III

- To apply cytokine decoy EVs for antagonizing IL-6 trans signalling pathway to promote myogenesis *in vitro*.
- To investigate the therapeutic efficacy of IL-6 trans signalling axis targeting decoy EVs in a mouse model of DMD.

3 MATERIALS AND METHODS

3.1 CELL CULTURE

In research article I, for screening of different EV engineering designs, HEK293T (Human embryonic kidney) cells transfected with respective constructs were used as an EV source. For *in vitro* uptake studies, Huh7 (human hepatocellular carcinoma) and B16F10 (mouse melanoma) cells were used. For *in vivo* experiments, human cord blood MSCs and HEK293T stably transduced with EV labelling construct were used as an EV source.

In research articles II and III, for screening of various EV display constructs, HEK293T cells transfected with respective constructs were used as an EV source. For measuring *in vitro* efficacy of cytokine decoy, HEK293T-based cytokine reporter cell lines were used. For measuring myogenesis, C2C12 (mouse myoblast) cells were used. For the *in vivo* experiments, bone marrow-derived MSCs stably transduced with different cytokine display constructs were used as an EV source.

Importantly, cells were cultured in serum-free conditions for EV production. The culturing conditions of each aforementioned cell line are described in detail in the individual research article.

3.2 PLASMID CONSTRUCTS AND CLONING

For paper I, codon-optimized DNA sequences encoding for all tetraspanins and luciferases were synthesized as linear dsDNA fragments (IDT), fragments were then cloned downstream of the CAG promoter in the pLEX plasmid backbone. In papers II and III, for TNFR1 display constructs, encoding sequences were amplified from cDNA and cloned downstream of CMV promoter into pEGFP-C1 plasmid backbone. For IL-6ST display constructs, all constructs were synthesized (Gen9) and were further cloned into the pLEX backbone downstream of the CAG promoter.

3.3 EV PURIFICATION

For all three research articles, a similar purification methodology was used. Briefly, conditioned medium (CM) was subjected to multiple low-speed centrifugation steps (500 × *g* for 10 min, 2000 x g for 20 min) to remove larger particles and cellular debris. Supernatant recovered was then filtered through 0.22 µm cellulose acetate membrane filters to remove any remaining larger particles. The precleared CM was then subjected to ultrafiltration either using Amicon Ultra-15 100 kDa spin filter at 4000 x g for 30 min or using TFF with a cut-off of 300 kDa to further concentrate the CM. EVs were further purified by SEC or bind elute-SEC, and EV fraction was further concentrated using an Amicon Ultra 10 kDa spin filter at 4000 x g for 30 mins.

3.4 EV CHARACTERIZATION

3.4.1 NTA

Nano tracking analysis in papers I, II and III were performed using the NS500 nanoparticle analyzer (Nano Sight) to measure the size distribution and concentration of EVs based on the Brownian motion of the particles.

For all measurements, the camera level was set at 10–13, and five 30 s videos for each sample were captured. All post-acquisition settings were set on auto. The detection threshold was fixed either at 6 or 7. Samples were diluted in PBS between 1:500 to 1:5,000 dilution factor to achieve a particle count of between 2×10^8 and 2×10^9 per ml. The camera focus was adjusted accordingly based on the user interpretation.

3.4.2 Multiplex bead-based assay

The EV surface proteome characterization in papers I and II, was assessed by MACSPlex Exosome kit (Miltenyi Biotec). Samples were prepared as per the manufacturer recommendations. Next, samples were analysed on a Flow cytometer (MACSQuant Analyzer). For data analysis, FlowJo software (v10, FlowJo LLC) was used to calculate the median fluorescence intensity for all 39 capture bead subsets. Data were then normalized to non-EV buffer or media controls.

3.4.3 Western blot analysis

The EVs in all three papers were assessed for the presence of prominent EV markers and the absence of apoptotic body markers. Furthermore, western blot was also used to assess the loading of the engineered fusion protein in the EVs. Briefly, cells were first lysed with RIPA buffer on ice. The cell lysate was then cleared of all debris by high-speed centrifugation. Next, cell lysate or EVs were denatured using SDS buffer (0.5 M dithiothreitol, 0.4 M sodium carbonate (Na_2CO_3), 8% SDS, and 10% glycerol), followed by incubation at 65˚C for 5 minutes. The samples were loaded onto a NuPAGE Novex 4–12% Bis-Tris Protein Gel (Invitrogen, Thermo Fisher Scientific) and ran for 2 hours at 120 V in NuPAGE MES SDS running buffer (Invitrogen, Thermo Fisher Scientific). Next, the proteins on the gel were transferred to an iBlot nitrocellulose membrane using the iBlot system (Invitrogen, Thermo Fisher Scientific). Next, the membrane was blocked using Odyssey blocking buffer (LI-COR) for 60 min at room temperature. After blocking, the membrane was incubated either overnight at 4°C or 1 hour at room temperature with primary antibody solution in Odyssey blocking buffer. Antibodies used and dilution factor are as follows: 1:1000 dilution for anti-Alix (ab117600, Abcam), anti-Tsg101 (ab30871, Abcam) and anti-NanoLuc (Promega); 1:2000 dilution for anti-CD9 (ab92726, Abcam), anti-calnexin (ab22595, Abcam), anti-His (34660, Qiagen), anti-hTNFR (ab19139, Abcam), anti-mGp130 (R&D, AF468) antibodies. After primary antibody staining, the membrane was washed with PBS supplemented with 0.1% Tween-20 and incubated with the corresponding secondary antibody (LI-COR) for 1

hour at room temperature. The membrane was washed first with PBS-Tween20 and lastly with PBS and visualized on the Odyssey infrared imaging system (LI-COR).

3.4.4 Transmission electron microscopy

For immunogold labelling, purified EVs were incubated with 1 µL of 1% BSA diluted in PBS for 5 min. Primary antibodies (2 µL of 1 mg/mL anti-hTNFR; Abcam, ab19139, 2 µL of 0.2 mg/mL anti-mGp130; R&D, AF468) were added and incubated for 45 min. Next, 2 µL of protein-A-conjugated 10 nm gold nanoparticles (BBI Solutions) were added and incubated for 45 min. For imaging, either Immunogold labelled EVs or purified EVs were added onto glow-discharged formvar-carbon-coated grids (TED Pella Inc.) for 1 min. Next, the grids were blotted dry with filter paper and stained with 2% uranyl acetate (UA) for 1 min. EVs were imaged with an FEI Tecnai 10 transmission electron microscope at an accelerating voltage of 100 kV.

3.4.5 Single-EV imaging flow cytometry

Engineered EVs were analysed with the ImagestreamX MkII instrument (Amnis/Luminex). The EVs samples were stained with either TNFR1 or IL6ST antibodies. All analyses were performed using the ×60 objective and a flow rate of 0.38 µL/min. Acquired data were analysed using Amnis IDEA software and Flowjo.

3.5 IN VITRO ASSAYS

3.5.1 Bioluminescence assay

For the detection of NanoLuc luciferase activity in EVs, purified EVs solution or CM was added into white-walled 96-well plates along with 30 µL Nano-Glo substrate (Nano-Glo Luciferase Assay System: Promega), as per the manufacturer instructions. For the detection of ThermoLuc and Firefly luciferase activity, purified EVs solution or CM (lysed in 0.1% triton X-100) was added into white-walled 96-well plates along with 30 µL luciferin substrate (Firefly Luciferase Assay System: Promega), as per the manufacturer instructions. The luciferase intensity in each well was immediately measured using a GloMax® 96 Microplate Luminometer machine (Promega).

3.5.2 Cytokine potency assay

NF-κB reporter (Luc)-HEK293 cells (BPS Bioscience, 60650) were cultured as per the manufacturer recommendations. For the assay, cells were seeded onto a 96-well plate with culture medium and incubated at 37 °C under 5% CO_2. After 24 hours, the cells were treated with or without decoy EVs, and with hTNF-α (5 ng /mL; NordicBiosite). Then, 6 hours after treatment, the cells were lysed using 0.1% triton X-100 in PBS (Sigma-Aldrich) and analysed by firefly luciferase assay. For measuring IL-6 activity, HEK-Blue IL-6 Cells (Invivogen, hkb-hil6) were cultured and used as proposed by the manufacturer. For the assay, cells were seeded in a 96-well plate with culture medium and incubated at 37 °C under 5% CO_2. After

24 h, the cells were treated with or without EVs and 5 ng/mL IL-6–IL-6R complex (hyperIL-6). 6 hours later, 20 µL cell culture supernatant was used to evaluate secreted alkaline phosphatase activity using QUANTI Blue reagent (Invivogen).

3.6 IN VIVO EXPERIMENT

All *in vivo* experiments related to this paper were performed under ethical permission granted by authorities in Sweden, Germany, Belgium, Italy and United Kingdom.

3.6.1 Paper I

For *in vivo* EV pharmacokinetic studies, female NMRI mice or C57BL6/j mice with a bodyweight of around 20 g, were injected with luciferase labelled EVs. Animals were sacrificed at different time points as per the experiment. Blood was collected by heart puncture into PST-tubes (BD Biosciences) and processed to retrieve plasma according to the manufacturer's instructions. The animals were then perfused with cold PBS and different organs were harvested and stored in 2 mL Eppendorf tubes at −80°C until further use.

For NanoLuc measurement, tissues were lysed in 1 mL 0.1% Triton X-100 in PBS using a Qiagen Tissue Lyser II. Tissue lysate was then further diluted in 0.1% Triton X-100 by 10-fold. Diluted tissue lysate was added into white-walled 96-well plates along with 30 µL Nano-Glo substrate (Nano-Glo Luciferase Assay System: Promega). The luciferase intensity in each well was immediately measured using the GloMax® 96 Microplate Luminometer machine (Promega). For ThermoLuc measurement, animals were imaged by IVIS Spectrum (Perkin Elmer) after intraperitoneal administration of 150 mg/kg D luciferin.

3.6.2 Paper II

3.6.2.1 Systemic inflammation model

Systemic inflammation was induced by intraperitoneal administration of LPS in female C57BL/6 mice. For therapeutic intervention, engineered EVs were injected intravenously through the tail vein after disease induction. All animals were scored and weighed daily after disease onset.

3.6.2.2 EAE (experimental autoimmune encephalomyelitis) model

For EAE induction, female C57BL/6 mice were immunized by subcutaneous injection of MOG35-55-CFA emulsion, distributed to three different locations. This was followed by intraperitoneal injections of pertussis on day 0 and day 2 post immunization. All animals were scored and weighed daily after disease induction. For therapeutic intervention, engineered EVs were injected either subcutaneous or intravenous post immunizations.

3.6.2.3 TNBS-induced colitis model

For TNBS-induced colitis induction, female BALB/c mice were pre-sensitized with peritoneum skin application of TNBS solution. Seven days later, TNBS-colitis was induced in pre-sensitized mice by intrarectal administration of 30 µL TNBS + 42.1 µL 95% ethanol + 27.9 µL H_2O per mouse. All animals were daily scored and weighed after disease induction. For therapeutic treatment, EVs were injected intravenously on day 1 post disease induction.

3.6.6 Paper III

Wild-type C57BL6/J or mdx/IL6 mice were treated with engineered EVs twice per week for two weeks intravenously. Animals were monitored daily post therapeutic intervention.

4 RESULTS AND DISCUSSION

4.1 PAPER I

The extracellular space is rich in various nanoparticulate species ranging from big apoptotic bodies to exosomes, lipoprotein complexes, ribonucleoprotein complexes, etc. Among these, vesicles like exosomes and MVs represent an essential component for intercellular communication. Therefore, tools for characterizing their spatiotemporal properties are needed to dissect their biological input in pathophysiology, development, and biotherapeutics delivery. The present spectrum of EV tagging methods fails to achieve specific labelling and lacks sensitivity. Furthermore, tools like lipophilic dyes alter EVs properties and may not reflect the true fate of an EV. Here, in paper I, we have explored bioluminescence labelling of EVs by tethering luciferase enzymes to EV proteins in order to achieve sensitive and specific detection of EV subtypes both *in vitro* and *in vivo*.

Others have previously utilized bioluminescence labelling; however, these approaches lack sensitivity due to the low quantum yield of luciferase enzymes and poor labelling efficiency of EVs. To address these two limitations, we first sought to identify an efficient EV engineering strategy, allowing us to load a high number of reporter proteins into the majority of EV subtypes by endogenous engineering. We evaluated five different EV sorting domains for loading EGFP into EVs. We identified three different EV specific tetraspanins CD63, CD81 and CD9, as our lead candidates that could label between 15-25% of the EVs population. These results were in line with our previously published work and previous observations by others on using CD63, CD81 and CD9 for endogenous EV engineering, although levels may differ across the experiments due to differences in transfection efficiency of the cells[41,181,254].

Next, we aimed to identify potential luciferase enzymes for EV labelling. For this, five different luciferases enzymes from literature were selected based on signal stability, biological half-life, and compatible emission spectrum for *in vivo* applications. To assess the efficiency of bioluminescent labelling, EVs purified from the cells either transfected with constructs expressing free luciferase enzyme or N/C- terminally fused to CD63 were subjected to luciferase assay. As expected, CD63 fusion, specifically C-terminal fusion, showed the highest bioluminescent labelling per particle. We identified NanoLuc and ThermoLuc as our lead candidates for EV labelling. In addition to showing efficient labelling as compared to other luciferase candidates, these were also stable over time at 37˚C in FBS for up to 4 and 2 days respectively, upon encapsulation into EVs. Furthermore, both ThermoLuc and NanoLuc engineered EVs showed linearity in signal intensity vs the dilution factor, with a detection limit as low as 5000 EVs for the NanoLuc engineered EVs. Notably, the surface proteome was unaltered on the CD63-luciferase engineered EVs as determined by a multiplex bead-based EV flow cytometry panel.

Based on the above-described findings, we next utilized bioluminescent labelling for tracking *in vitro* EV release and *in vitro* EVs uptake. Overall, we observed no difference in the release of different EV subtypes (CD63, CD9 and CD81) but interestingly, culturing cells in optimized serum-free conditions enhanced EV release as compared to serum-containing medium. This phenomenon was further dissected in a separate study and we identified upregulation of the non-ESCRT pathway as a major driver for the enhanced EV release in serum-free conditions[72]. For *in vitro* uptake, purified ThermoLuc EVs showed a dose-dependent uptake both in B16F10 melanoma and Huh7 hepatocellular carcinoma cells.

Given the fact that as low as 5000 NanoLuc EVs can be detected along with high serum stability, the application of NanoLuc labelling is ideal for *in vivo* biodistribution studies. Therefore, we sought to determine the pharmacokinetics of EVs produced from a therapeutically relevant cell source, i.e., cord blood MSCs. Mice were injected with $1x10^{11}$ CD63 NanoLuc cord blood MSC EVs and biodistribution of EVs was determined at different time points ranging from 5 minutes to 24 hours. Interestingly, 90% of the injected dose was cleared from the plasma and accumulated in all the organs analysed in the study within 5 minutes post-injection, showing a rapid *in vivo* uptake in the tissues. As expected, the liver and spleen were the primary accumulation sites, accounting for 99% of the detected signal at 5 minutes post-injection.

Interestingly, the NanoLuc signal peaked at 5 minutes post-injection and showed a rapid decline over time to no or very low signal at 24 hours post-injection. This could be due to cargo degradation after being taken up in the tissues. Notably, the degradation rate of the signal was different across all tissues, which could be due to differences in cell type taking up EVs in these tissues. Overall, these results indicate that EVs show a rapid *in vivo* clearance profile, with a plasma half-life of 1.2-1.3 minutes. Importantly, these results correlate well with the previously reported *in vivo* uptake profile of EVs[280]. Furthermore, different systemic administration routes such as intraperitoneal, intravenous, intracardiac and intracarotid administration yielded similar EV *in vivo* biodistribution profiles, clearly indicating that EVs association with certain plasma components determine the *in vivo* biodistribution profile. In contrast, the local administration route showed very little systemic distribution of EVs.

Although NanoLuc labelling allows for sensitive quantification of EVs, non-invasive imaging is challenging due to the poor *in vivo* distribution of NanoLuc luciferase substrate. Therefore, for non-invasive imaging of EV biodistribution, we applied ThermoLuc labelling. Similar pharmacokinetics were also observed with ThermoLuc EVs, with the majority of EVs being taken up within 30 seconds of administration by the lung, liver and spleen, followed by degradation of the cargo over time.

In conclusion, these two enzymes serve as an excellent tool for tracking EVs *in vitro* and *in vivo*. Importantly, due to endogenous labelling of the EVs with a luciferase protein, label will reflect the integrity of the protein cargo of EVs, which may resonate the true biological half-life of EVs. With this level of sensitivity and the ability to reflect the spatiotemporal

properties of an EV *in vivo*, this labelling system have great applicability in exploring EV engineering application for *in vivo* targeting and plasma half-life determination.

4.2 PAPER-II & III

Besides transferring biomolecules intracellularly, EVs also possess the natural ability to sequester bacterial toxins by surface display of receptors[192]. Inspired by this, in these two studies, we have adapted this novel property of EVs by displaying cytokine receptors without signalling domains to sequester pro-inflammatory cytokines.

To achieve this, a novel engineering method needed to be devised as tetraspanins which are often used for EV engineering does not allow for efficient surface display due to the closed extracellular topology of the protein. To this end, we designed a range of constructs where either TNFR1 (Receptor for TNF-α) or IL-6ST (Receptor for IL-6/IL-6R) were fused with a range of EV sorting proteins or their respective domains. To evaluate the engineering efficacy across the different designs, EVs were assessed based on their ability to sequester cytokines and inhibit their bioactivity in an *in vitro* reporter assay for TNF-α (NFkB-Luc) or IL-6/sIL-6R(STAT3-SEAP). EV display constructs with the N terminal domain of syntenin as a sorting domain outperformed all the other designs in decoying either TNF-α or IL-6/sIL-6R complexes. To further improve these receptors' loading and binding affinity towards their respective cytokine by mimicking the natural oligomeric receptor state *in situ*, we introduced a multimerization domain at various positions in the construct. For TNFR1 based constructs, a trimerization domain from T4 fibritin protein of T4 bacteriophage was used, and for IL-6ST based constructs, a dimerization domain from GCN4 protein was added. As expected, the addition of multimerization domains in both TNFR1 and IL-6ST further improved the potency of engineered EVs. Notably, in an *in vitro* reporter assay, TNFR1 display EVs showed 10-fold lower IC50 values for TNF-α blockade than clinically approved biologic against TNF-α.

Next, we evaluated what the impact of EV engineering strategy could have on the inherent properties of EVs. In terms of the surface proteome, engineered EVs surface profile was similar to MSC ctrl EVs. We additionally evaluated the *in vivo* toxicity of these EVs in a multiple-dose and dose-escalation study in a murine disease model for DMD and observed no differences in haematological (WBC count, Neutrophils, Monocytes, B cells, NK cells, DCs and T cells) and physiological (body weight and spleen weight) parameters compared to a saline-treated animal. Overall, these results indicate that this EV engineering strategy allows for efficient surface display of biotherapeutics without altering the surface proteome and inherent properties of EVs.

With promising *in vitro* potency of these engineered EVs, we aimed to evaluate the *in vivo* efficacy of cytokine decoy EVs. To develop an initial proof of concept, we started our evaluation first in the LPS-induced systemic inflammation model. Simultaneous LPS challenge and systemic delivery of TNFR1 or IL6ST decoy EVs showed a significant

increase in the survival rate. Moreover, similar results were also observed in a subsequent study where TNFR1 decoy EV treated mice showed 100% survival at 60h after disease induction. In contrast, clinically used drugs against TNF-α exhibit only 25% survival at 60 hours post disease induction. Although we observed improved survival in these mice, the decrease in weight loss could not be improved. Therefore, we hypothesized this could be due to a lower dose with respect to the severity of the disease. Thus, in another subsequent study with increased dose, a significant decrease in the weight loss was observed compared to control EV treatment.

After establishing the first *in vivo* proof of concept, we next aimed to utilize the unique ability of EVs to cross biological barriers such as the blood-brain barrier in the CNS in conjunction with our cytokine decoy EVs. To this end, we evaluated the therapeutic efficacy of cytokine decoy EVs in a neuroinflammation model. Repeated administration of TNFR1 decoy EVs on days 7, 10 and 13 showed a significant decrease in the clinical score on day 16 (humane endpoint) compared to control EVs and saline treatment. Furthermore, treatment with TNFR1 decoy EVs showed a significant decrease in TNF-α and IL-6 gene expression in the spinal cord of the animals on day 16. Besides, a similar therapeutic outcome in regard to EAE clinical score was also observed after repeated treatment of IL-6ST EVs in a subsequent study.

Next, we evaluated the possibility of simultaneous display of two different cytokine receptors on the EV surface. Co-expression of both designs - TNFR1 and IL-6ST, in the producer cells resulted in up to 40% of double engineered EVs. Strikingly, double decoy EVs showed similar *in vitro* potency upon benchmarking it against the single decoy counterpart, hence indicating that expression of the transgene is primarily the limiting factor. After observing robust *in vitro* potency, we next evaluated double decoy EVs in an intestinal TNBS-induced colitis model which mimics Chron's disease in humans. Single treatment of double decoy EVs 24 hours after the disease onset showed a significant dose-dependent increase in the weight recovery of the animals.

Moreover, double decoy EVs performed better than the combination of clinically approved drugs against TNF-α and IL-6R. Importantly, in study I, we observed rapid clearance of EVs from plasma and tissues. However, these studies were performed in wild type mice and in a diseased state, EV biodistribution could be drastically different. To address this, we compared the *in vivo* biodistribution of the EVs in wild-type mice and mice with intestinal inflammation. Strikingly, we saw a 300-fold increase in EV accumulation in the liver and up to 100-fold in the intestine. Furthermore, in another ongoing study, we have observed up to 10^6 fold increase in plasma levels of exogenous EVs in mice with systemic inflammation (Pavlova *et al.*). Although the exact mechanism behind this is unknown and warrant further investigation, it provides a plausible explanation as to why decoy EVs are so active in inflammatory settings.

Apart from systemic inflammatory diseases, inflammation also plays a critical role in muscle pathologies. Recent evidence has suggested the essential role IL-6 plays in driving inflammation which can potentially inhibit skeletal muscle expansion and myogenic potential[330]. In light of this, we explored the potential application of IL-6ST decoy EVs in muscle related pathologies. DMD is one such muscle pathology where inhibition of IL-6 signalling has shown great therapeutic promise. DMD is an autosomal recessive disease caused by the of dystrophin function, a structural protein involved in maintaining muscle integrity and deficiency of it leads to membrane damage, inflammation, and degeneration of skeletal muscle. Furthermore, pro-inflammatory cytokines upregulate STAT3 signalling to control muscle differentiation and expansion[331]. Therefore, targeting the IL-6-STAT3 signalling axis should enhance muscle function. First, we evaluated the efficacy of IL-6 inhibition in an *in vitro* myogenic differentiation assay. Treatment of myoblasts with IL-6/sIL-6R complexes markedly decreased the myotube formation by activating STAT3 signalling. Treatment with IL-6ST decoy EVs significantly downregulated IL-6/sIL-6R mediated STAT3 phosphorylation in both myoblasts and myotubes. Furthermore, IL-6ST decoy EV treatment significantly enhanced the myogenic index of myotubes compared to control EV or mock treatment.

After establishing the *in vitro* potency of the EVs, IL-6ST decoy EVs were repeatedly administered twice per week for two weeks in DMD mice overexpressing IL-6. Remarkably, IL-6ST decoy EV treatment significantly decreased the STAT3 phosphorylation in Gastrocnemius by up to 70% compared to control EV treated mice. Furthermore, similar inhibition was also observed in the Quadriceps muscles of the mice, where STAT3 phosphorylation was downregulated by 80% compared to control EV-treated mice. These results showcase EVs unique ability to achieve an activity in hard-to-reach tissues, such as muscles. Current efforts are focused on understanding the myogenic potential of satellite cells *in vivo* after treatment with IL-6ST decoy EVs. Furthermore, displaying a muscle-targeting peptide can potentially enhance the bioavailability of decoy EVs in muscle to further enhance the therapeutic effect *in vivo*.

In conclusion, this platform allows for an efficient mean to display biotherapeutic cargo on the surface of EVs. Moreover, decoying biologics using EVs proved to be a viable strategy, as they can achieve a dynamic distribution *in vivo* in contrast to protein biologics.

5 FUTURE PERSPECTIVES

The field of EVs has seen immense progress in the past few decades, from being regarded as garbage bags to now be considered as essential mediators in intercellular communication. Owing to their unique ability to transfer macromolecules across cells and biological barriers, EVs are considered a rising star in the field of drug delivery. Notably, EVs outcompetes the majority of the synthetic delivery vectors in terms of efficacy, extrahepatic delivery, and toxicity. Several clinical trials utilizing EVs as a therapeutic intervention have demonstrated their biocompatibility, however, they have failed to achieve the primary end point efficacy, highlighting considerable gaps in knowledge about EVs.

In spite of an exponentially increasing amount of information being reported about EVs, the research is still in its early stage, and various technological and biological limitations need to be addressed. In terms of technical limitations, most of them are associated with the manufacturing, purification, and quantification of EVs. Therefore, technologies that allow for large-scale production of highly pure EVs without affecting their integrity and properties should be developed. Furthermore, quantification is one of the critical aspects which lacks standardisation across the field. Therefore, alternative ways of quantifying EVs should be explored, such as EV potency assays or quantifying EVs based on bioactive cargo inside the EVs.

On the biological aspect of EVs, future work on understanding EVs heterogeneity and identifying EV subtypes and their physiological role may aid in our understanding. This is an important aspect, as the EV subtype may be remarkably different in content and may exert diverse biological effects. Furthermore, it is evident that cell source for EV production can have dramatic effect on *in vivo* cellular tropism of EVs and diverse immunomodulatory profile. Therefore, characterising those critical components in the EVs driving this heterogeneous response may aid future therapeutic applications. However, despite various technological hurdles, we have come a long way from the dust, and with this pace of development, EV based therapeutics will find their place on the shelf.

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