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# Extracellular vesicles as drug delivery systems: Why and how?

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ABSTRACT

Over the past decades, a multitude of synthetic drug delivery systems has been developed and introduced to the market. However, applications of such systems are limited due to inefficiency, cytotoxicity and/or immunogenicity. At the same time, the field of natural drug carrier systems has grown rapidly. One of the most prominent examples of such natural carriers are extracellular vesicles (EVs). EVs are cell-derived membranous particles which play important roles in intercellular communication. EVs possess a number of characteristics that qualify them as promising vehicles for drug delivery. In order to take advantage of these attributes, an in-depth understanding of *why* EVs are such unique carrier systems and *how* we can exploit their qualities is pivotal. Here, we review unique EV features that are relevant for drug delivery and highlight emerging strategies to make use of those features for drug loading and targeted delivery.



**Contents**



## 1. Introduction

Over the course of decades, various synthetic nanoparticulate delivery systems have emerged and been utilized to improve the pharmacokinetic and pharmacodynamic profile of therapeutics. When carried by a delivery system, clearance and tissue distribution profile of a therapeutic are mainly governed by the characteristics of the vehicle rather than the physicochemical properties of the drug molecule [1–3]. For this reason, synthetic drug delivery systems have been exploited in order to enhance drug efficacy and therapeutic index, while concomitantly minimizing drug toxicity and off-target side effects [4]. Liposomes, micelles, dendrimers, nanocapsules, nanosponges and peptide-based

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nanoparticles are amongst the most prominent examples of these synthetic drug delivery systems [1,5]. Of these, the oldest and most extensively studied vehicle is the liposome [6]. Doxil® for example, the first liposomal formulation on the market, was approved by the Food and Drug Administration (FDA) already in 1995 [6].

Liposomes are composed of a biocompatible lipid bilayer, which can accommodate hydrophobic drugs, and an internal aqueous phase in which hydrophilic drugs can be captured [7,8]. Despite the advantages that liposomal formulations offer for therapeutic delivery, there are many hurdles that still exist for the delivery of drugs to target organs. For example, the rapid clearance of liposomes via the reticuloendothelial system (RES), and their accumulation in liver and spleen limits the dose that reaches the target site [7,9]. Moreover, some studies suggest that the accumulation of liposomes in macrophages, especially at higher doses, can influence their phagocytic activity, leading to immune suppression and hampering the clearance of bacteria from the bloodstream [7,10,11]. An additional drawback of using liposomal formulations is the activation of an acute hypersensitivity reaction: complement activation–related pseudoallergy (CARPA). This acute allergic reaction, which triggers the release of histamine, tryptase and leukotrienes, results in the discontinuation of treatment in some cases of hypersensitive individuals [7]. One of the many ways to overcome the limitations of liposomes is by diverting to the utilization of natural carrier systems for the delivery of therapeutics. Recently, progress in the field of biological or bioinspired drug carriers has been growing expeditiously. Amongst the continually expanding fields of interest is the field of extracellular vesicles (EVs). EVs are cell-derived membranous structures that are capable of transporting various active biomolecules from producer cells to recipient cells, thereby changing the physiology of the recipient cells [12,13]. Such abilities have drawn a great deal of attention towards EVs for therapeutic application, and as a prospective vehicle for the delivery of therapeutics that could overcome issues related to liposomes and other synthetic drug delivery systems [14].

## 2. Extracellular vesicles

Extracellular vesicles are phospholipid bilayer-enclosed vesicles secreted from all cell types and can therefore be found in tissue culture supernatants as well as biological fluids such as blood, saliva, breast milk, cerebrospinal fluids and malignant ascites [15–17]. EVs make up a heterogeneous population of particles that are generally classified into three distinct populations based on their biogenesis: exosomes, microvesicles and apoptotic bodies [17]. Exosomes are formed as a result of inward budding of the limiting membrane of endosomes to form multivesicular bodies (MVBs). Subsequently, exosomes are released into the extracellular space by fusion of MVBs with the plasma membrane. Following release from the cell surface, exosomes can interact with the extracellular matrix, or elicit a response in cells within the microenvironment or at a distance [18,19]. On the other hand, microvesicles arise from direct outward budding of the plasma membrane, producing a population of EVs that is heterogeneous in size. Apoptotic bodies are also generated from the cell surface, although they are only released by dying cells during cell fragmentation [17–20]. Exosomes have a size ranging from 40 to 120 nm, while microvesicles have a size of 50–1000 nm [21,22]. As a result of their overlapping sizes, surface markers, and the absence of proteins that are restricted to specific populations, it has been challenging to distinguish between exosomes and microvesicles; therefore, all different types of vesicles will be referred to as EVs in this review.

EVs function as a carrier for various biomolecules, such as proteins, lipids, DNA and a repertoire of RNA species [10]. Some lipids are more enriched in EVs compared to the plasma membrane, e.g. cholesterol, phosphatidylserine (PS), glycosphingolipids, sphingomyelin (SM) and unsaturated lipids; however, the relative abundance of specific lipids differs amongst EVs from different producer cell types [23,24]. A collection of proteins have been confirmed to be enriched in EVs, including both cytosolic and membrane proteins, e.g. annexin II and heat shock proteins, MHC class II complexes, integrins and tetraspanins, in addition to ALG-2-interacting protein X (Alix), tumour susceptibility gene 101 (TSG101), as well as cell-specific proteins that may have an influence on EV function [23]. These cell-specific proteins are dependent on the producer cell. For example, EVs derived from B-lymphocytes are enriched in MHC-II peptides [25], while EVs derived from glioma cells carry EGFRvIII [26].

EVs possess features that qualify them as a potential avenue for therapy and as a drug delivery system, as shown in Fig. 1. For example, EVs carry and protect a wide array of nucleic acids and seem intrinsically capable of their functional delivery into recipient cells [27]. Another of these acclaimed features is their intrinsic stability in circulation due to their negatively charged surface and their ability to avoid the mononuclear phagocytic system (MPS) by exhibiting the surface protein CD47 [10]. Contrastingly, other studies show that EVs have similar clearance kinetics as liposomes [28], or that they are even cleared more rapidly following intravenous injection [29,30]. These seemingly contradictory results may be a consequence of differences in EV cell source, isolation procedure (known to affect EV integrity and biophysical properties [31,32]), or specific EV protein/lipid surface profile, although the underlying mechanisms remain to be elucidated. Moreover, EVs may have the capacity to cross biological barriers [33–35], exploit endogenous intracellular trafficking mechanisms and trigger a response upon uptake by recipient cells [36]. In addition, they may display inherent targeting properties that are dictated by their lipid composition and protein content [36]. Studies have shown that specific progenitor cell derived EVs convey biological cargo that promotes angiogenesis and tissue repair and modulates immune functions [37]. As such, these EVs exhibit a promising source of acellular therapy for various conditions, which can be taken advantage of by further engineering these EVs for the delivery of therapeutics. Importantly, EV producer cells can be exploited for the manufacturing of biological therapeutics as well as targeting ligands to be loaded in- or onto EVs. These advantages open the door for a drug delivery vehicle that can potentially compensate for the drawbacks of synthetic delivery systems. However, in order to utilize these advantages for the benefit of drug delivery via EVs, it is of great

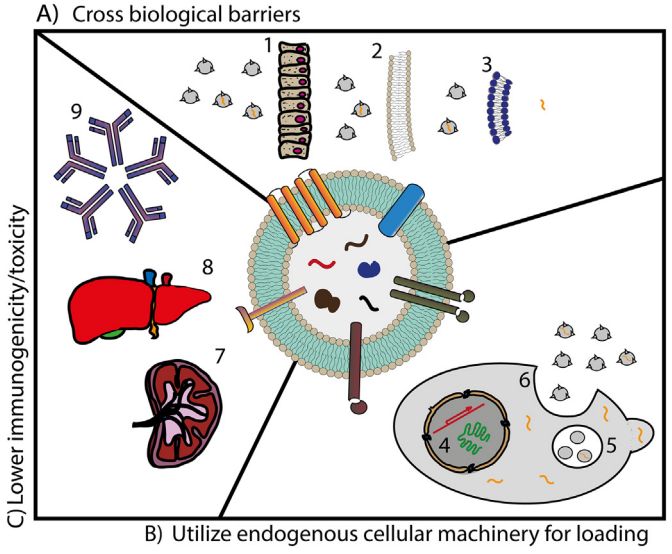


**Fig. 1.** Proposed unique features of extracellular vesicles. EVs are believed to (A) be able to cross various biological barriers including tissue barriers (1), plasma membranes (2), and deliver cargo across endosomal membranes (3), (B) utilize endogenous cellular machinery for manufacture/loading of different cargo through the production inside the nucleus (4), which is then loaded inside MVBs or at the plasma membrane (5), and eventually released in the extracellular space inside EVs (6), and (C) display lower toxicity in spleen (7), and liver (8) as well as reduced immunogenicity (9).

importance to first understand (1) *why* EVs may be suitable drug delivery vehicles and what their potential benefits are over synthetic vehicles, and (2) *how* EVs can be engineered and pharmaceutically developed in such a way that clinical usage of EVs as drug carriers may become a reality.

## 3. Why: Endogenous cellular sorting and packaging

One potential advantage of using EVs for delivery of biotherapeutics over synthetic carriers is that endogenous cellular machinery can be used to produce the desired cargo, and sort it inside EVs, as manufacturing, storage and loading of such biotherapeutics can be challenging. This is especially true for protein-based therapeutics, whose stability is sensitive to changes in temperature, solvent and pH. In order to exploit this advantage, a detailed understanding of the underlying mechanisms is needed. Here, we focus on protein and RNA cargo in EVs, as these biotherapeutics are the most widely studied as potential therapeutics.

EVs contain a mixture of proteins that are integrated in, or attached to, their membranes or present in their intraluminal space. Different pathways for EV biogenesis are reflected in their protein composition [38]. For example, as compared to cells, exosomes are enriched in components of the endosomal sorting complex for transport (ESCRT) machinery and its accessory proteins, including for example Alix and TSG101. These proteins are therefore often used as exosome marker proteins [38,39]. ESCRT plays a major role in cargo clustering during intraluminal vesicle (ILV) biogenesis [40]. ESCRT consists of four complexes (ESCRT-0, -I, -II and -III), which have crucial roles in EV biogenesis and release. On the other hand, ESCRT-independent budding machinery has also been found to be involved in the biogenesis of EVs [40]. For example, an alternative pathway employs ceramide for the generation of another population of ILVs, thereby resulting in secretion of exosomes with different characteristics [41]. Other proteins reported to be abundant in EVs include heat shock proteins (e.g. Hsp70 and Hsp90), lysosomal-associated membrane proteins (e.g. Lamp2a, Lamp2b), cytoskeletal proteins (e.g. actin, tubulin and cofilin) and tetraspanin proteins (e.g. CD9, CD81, and CD63) [42]. In addition, Rab GTPases (e.g. Rab4, Rab11, and Rab27), annexin and flotillin that play a major role in vesicular trafficking and release are present [43]. Although exosomes and microvesicles have seemingly different protein profiles as a result of their different biogenesis pathways, no specific protein has been identified to discriminate between the two populations. Having a better understanding of EV proteomic profiles and how protein composition is influenced by various factors should enable a more efficient exploitation of engineering strategies for the delivery of therapeutics in the future.

Besides proteins, EVs also carry a multitude of coding and non-coding RNAs, including mRNA, miRNA, circRNA, tRNA, snoRNA, and piRNA [44,45]. The apparent enrichment of some specific RNAs in EVs compared to producing cells suggests that EV cargo loading is not a completely random process, but that, at least to some extent, an organized mechanism that orchestrates RNA sorting into EVs must exist. The exact mechanism that regulates the loading of RNAs into EVs is still poorly defined; however, the results of several studies imply that more than one mechanism is involved [46].

Some RNA-binding proteins have been identified to participate in the EV miRNA sorting machinery that is regulated through specific sequence motifs [46,47]. These proteins bind directly to specific miRNAs and enhance their loading inside EVs. For example, synaptotagmin-binding cytoplasmic RNA-interacting protein (SYNCRIP) in hepatocytes and sumoylated heterogeneous nuclear ribonucleoprotein (hnRNPA2B1) in lymphocytes recognise GGCU and GGAG motifs in specific miRNAs, respectively [46,47]. This suggests that conserved motifs in miRNA sequences control their compartmentalization inside vesicles through RNA binding proteins. This may be exploited by inserting these specific sequences in other, more cell-retained miRNA in order to increase EV loading [46]. In addition, the RNA binding protein Y-box protein 1 (YBX1) can physically interact with a specific miRNA (i.e. miR-223) through an internal cold shock domain and a highly charged C-terminus, and package it inside EVs; however, a specific sequence motif in the miRNA was not identified [48]. Other studies have also reported a role for YBX1 in binding various other small RNAs, including tRNA fragments and snoRNAs, and loading them inside EVs.

In addition to the packaging of specific miRNAs inside EVs, pathways for the trafficking of the bulk of miRNA cargo into EVs have also been revealed. A recent study described an effect of ADP-ribosylation factor 6 (ARF6)-GTP and Exportin-5 on the trafficking of pre-miRNA into tumour cell-derived microvesicles (TMVs) [49]. It was shown that activation of ARF6 leads to a global increase in the miRNA content within TMVs. A large-scale mapping of protein-protein interactions revealed that Exportin-5, a protein that is responsible for the transport of pre-miRNA and RNA-binding proteins from nucleus to cytoplasm, is an ARF6 binding partner. Additionally, a GTP-mediated interaction between Exportin-5 and ARF6, and their co-localization either in the vicinity of the nucleus or within budding TMV, indicates a role for Exportin-5 in the trafficking of pre-miRNA cargo from the nucleus to the site of TMV biogenesis. After TMV-mediated miR-21 transfer to recipient cells, which were engineered to express destabilized GFP contained a miR-21 binding site in its 3′UTR, GFP protein expression was decreased, indicating functional delivery of miR-21. This highlights the role of a specific pathway in the regulation of pre-miRNA, which may be employed for more efficient loading of therapeutics.

Moreover, EVs may comprise proteins that can bind to and sort their own mRNA. One such sorting mechanism that imitates viral RNA transfer is via a Group-specific antigen (Gag)-like protein, termed Arc [50,51]. Typically, during retroviral replication, Gag proteins form a capsid through multimerization to package viral RNA and transfer it to other cells. In a similar manner, the neuronal Gag-like protein, Arc, binds to its mRNA through ionic interactions in its N-terminus, resulting in subsequent self-assembly of capsids, RNA encapsulation in EVs and eventual transfer to neurons. It has also been observed that mRNA encapsulation by Arc can be nonspecific and therefore exploited for the co-transfer of highly abundant mRNAs from one cell to another via Arc-containing EVs [50].

Altogether, it is clear that several mechanisms play a role in the complex and multi-stage process of cargo sorting into EVs. Understanding the exact mechanisms involved in cellular sorting of cargo molecules would allow us to utilize these insights for the endogenous loading of various biotherapeutics into EVs, in order to use these EVs for therapeutic delivery. Unfortunately, given the apparent EV heterogeneity depending on biogenesis, method of isolation, and cell source, loading methods may have to be optimized for each producer cell type and desired therapeutic cargo individually.

## 4. Why: Intrinsic ability to cross physical barriers

One of the major difficulties that conventional synthetic delivery systems face is their inability to efficiently cross biological barriers, including tissue barriers, cellular barriers and intracellular barriers. In contrast, several studies have shown the ability of EVs to efficiently cross these biological barriers and induce functional changes in target cells as a result [33–35].

On a tissue level, as an example, EVs have been shown to be able to cross one of the most challenging barriers for delivery of therapeutics: the blood brain barrier (BBB). The BBB is a major hindrance to the functional delivery of therapeutic cargo to the brain for the treatment of central nervous system diseases, as it limits the passage of almost 98% of small molecule drugs [52]. Various studies have demonstrated a role for EVs in intercellular communication between neuronal cells, as EVs have been shown to conserve neuronal integrity [53], participate in synaptic plasticity [54], and maintain the brain milieu [55]. Moreover, emerging evidence highlights the capacity of EVs to transfer functional cargo across the BBB, from hematopoietic cells to the brain, at least

under inflammatory conditions [56]. Even though a number of studies support the ability of EVs to cross the BBB [33,35], the exact mechanism that governs this process remains ill-defined. Additionally, EVs have been shown to be able to cross the blood-cerebrospinal fluid barrier via the choroid plexus to reach the brain parenchyma [57].

On a cellular level, EVs are intrinsically able to interact with the plasma membrane through a variety of ligand/receptor interactions [58]. As a result, EVs seem to be internalized more efficiently than synthetic nanocarriers [59,60]. For example, it has been shown in a head-to-head comparison of cellular uptake that, while synthetic lipid nanoparticles accumulate into islands at the cell surface, with only a minor fraction being internalized, EVs enter cells within minutes of addition without accumulation at the cell surface first [61]. Thus, EVs seem to utilize endogenous mechanisms for cellular uptake, which may be advantageous for the delivery of therapeutics with intracellular targets. Importantly, however, it has been shown that EVs from the same cell source can interact distinctively with different recipient cell types [62]. Thus, intercellular communication and EV trafficking are dependent on the specific properties of the producer as well as recipient cells [63]. A better understanding of the reason for variation in cell-dependent intercellular EV transfer and uptake could improve EV-mediated delivery.

On an intracellular level, EVs seem to be mainly internalized via endocytosis, with different endocytic pathways identified for different cell types. The fact that EVs seem capable of delivering RNA cargo to recipient cells in a functional manner suggests that they utilize endogenous mechanisms for the delivery of cargo to the cytosol. Indeed, it has been hypothesized that EVs can fuse with endosomal membranes [64,65], although the mechanisms involved are unknown. Interestingly, it has been reported recently that EVs can fuse with membranes, but only at lower (i.e. endosomal) pH, and that proteins on both the EV and membrane side are important for this fusion event [66]. Developing more advanced technology that would allow more detailed investigation of the mechanisms underlying EV fusion and cargo release remains an important challenge.

Overall, it is clear that EVs can be considered a competent vehicle for the delivery of therapeutics, as EVs are able to efficiently cross different levels of biological barriers, deliver their cargo, and evoke a response in their recipient cells. However, the underlying mechanisms of these processes remain to be explored. Moreover, it should be taken into account that this promising quality to overcome various biological barriers could have limited value for some drug delivery applications as a result of the rapid clearance profile, at least for specific EV types, as discussed above.

## 5. Why: Safety profile

An important reason that EVs have been favoured as drug delivery vehicles is their potential to reduce the toxic effects that foreign substances have when introduced to the body. Due to their biological origin, EVs are likely to be minimally reactive to the immune system. For example, thousands of blood and plasma transfusions are being done every day, in which high numbers of EVs are being transferred to patients without apparent adverse effects. In contrast to virus-derived vehicles, or cell therapies, EVs are also relatively safe, as they are completely non-replicative and not mutagenic, which eschews regulatory concerns of adverse effects or neoplasia formation. These benefits have been confirmed by the low toxicity observed in in vivo trials of EV therapeutics. Below, we detail the findings of the preclinical and clinical trials undergone to date and discuss important considerations for EV safety.

The most comprehensive analysis of EV toxicity to date looked at the effects of engineered and wild type HEK293 EVs in immunocompetent C57BL/6 mice. The authors administered ten doses of approximately $10^{10}$ EVs IV or IP over 22 days, before assaying the animal's weights, cytokine concentrations, blood protein levels, spleen and blood cell compositions on day 23. The only values that deviated from PBS controls were slightly decreased levels of IP-10, MDC and MIP-1ß in two of the engineered EV groups, as well as modestly increased levels of circulating neutrophils in all but one of the EV administered groups [67]. These results have recently been extended to EVs derived from suspension cultured Expi293F cells administered to BALB/c mice. Although this work also observed an increase in overall WBC counts 24 h after injection, no notable toxicity or immune response was reported [68]. These results illustrate that EVs seem to be generally well tolerated, even when used xenogenically.

Importantly, these findings also seem to apply to other engineered cell types. For example, EVs derived from wild type foreskin fibroblasts (BJ cells), or those loaded with siRNA targeting a mutated version of KRAS, produced no significant blood cytotoxicity despite repeated injection over long time periods [69]. In a clinical trial, intrapleural injection of tumour cell derived EVs loaded with chemotherapeutics showed only low grade toxicity while producing clinical benefits [70]. Even when externally engineered, EVs do not seem to cause toxicity or inflammatory response. For instance, research involving the decoration of C2C12 cell EVs with anchor peptides and splice switching oligos for the treatment of muscular dystrophy showed no detectable toxicity or inflammation in the liver, kidneys or muscles of mice following IV injection [71]. These results suggest that EV engineering is a promising method for the delivery of therapeutic molecules. It should be noted however that fundamental differences exist in the activation of an acute hypersensitivity reaction (CARPA) between rodents and larger animals such as pigs [72], suggesting that safety studies performed in rodents should be interpreted with care.

Due to the wide application of MSC therapies, the bulk of clinical and preclinical trials involving EVs have utilized MSC EVs. Although a systematic review of MSC clinical trials found few toxic effects, the consistent appearance of fever in treated individuals suggests that cell therapy may be generally immunogenic [73]. Due to their reduced complexity and exposure of immunogenic proteins, such as MHC molecules, EV therapy may improve on this [74]. Since MSC paracrine functions have been shown essential for the therapeutic effects of MSCs [75], preclinical trials have used MSC EVs derived from swine, human and mouse sources, including umbilical cord, bone marrow, blood, adipose tissue, ESCs and iPSCs. These have been used in vivo for tissue regeneration and disease treatment in many systems, such as the respiratory, renal, hepatic, nervous, musculoskeletal and cardiovascular systems [76–83]. Unfortunately, very few of these studies have explicitly looked into the safety of their treatments, or run EV treatments in isolation, though they generally conclude them to have been well tolerated. Thus far, the clinical applications of bone marrow MSC EVs have targeted graft versus host disease and bone regeneration, while umbilical cord MSC EVs have been used to treat retinal lesions and chronic kidney disease [84–86]. Although the number of individuals treated have generally been low in these studies ([67,76,79,87]), no adverse effects have been attributed to the EV treatments used.

Another area where EVs have been clinically tested is as cancer vaccines. Such studies have used EVs derived from either tumour cells or primed dendritic cells to direct the immune system against specific tumours. Tumour cell EVs themselves have also attracted significant attention in this area, and one clinical trial has been completed and reported results operating on this principle [87]. When combined with adjuvants, this treatment elicited an anti-tumour immune response, without any significant toxicity as a result of tumour derived EV therapy. Dendritic cell derived EVs have also been applied clinically due to their ability to activate the natural killer cell immune response. In two trials involving the use of autologous dendritic cell EVs, none of the twenty-eight patients treated showed signs of adverse events above grade 2 toxicity [88,89]. In contrast, a study using heterologous interferon-gamma maturated dendritic cells loaded with MHC I- and II- cancer antigens found that 10% of patients exhibited grade 3 or 4 cytotoxicity [90]. All safety profiles for published clinical trials are listed in Table 1.

**Table 1**
Completed clinical studies of EV-based therapeutics.

| Indication | Phase | EV source | Safety profile | Purification method | Storage condition | | References |
|---|---|---|---|---|---|---|---|
| | | | | | Solution | Temperature | |
| Lung cancer | Phase I | Tumour cells, autologous | Mild to moderate AEs | Sequential differential centrifugation | 0.9% NaCl | Not clearly stated | [70] |
| Graft versus Host disease | Case study | MSC, allogenic | No AEs during treatment | PEG precipitation | 0.9% NaCl | −80 °C | [84] |
| Healing of macular holes | Pilot study | MSC, allogenic | Mild to moderate AEs | Sequential differential centrifugation | PBS | −20 °C (maximum storage 1 month) | [85] |
| Chronic kidney disease | Phase II/III | MSC, allogenic | Mild AEs | Sequential differential centrifugation | Medium M199 with 25 mM HEPES | −80 °C | [86] |
| Colon cancer | Phase I | Ascites, autologous | Mild to moderate AEs | UC - 30% Sucrose cushion – UC | PBS | −80 °C | [87] |
| Melanoma | Phase I | imDCs, autologous | Mild AEs | TFF - 30% Sucrose cushion - TFF | 20 mM Tris/1 mM MgCl2/5% sucrose/100 μg/mL human serum albumin (according to [91]) | Not clearly stated | [88] |
| Non-small cell lung cancer | Phase I | imDCs, autologous | Mild to moderate AEs | TFF - 30% Sucrose cushion - TFF | 20 mM Tris/1 mM MgCl2/5% sucrose/100 μg/mL human serum albumin (according to [91]) | Not clearly stated | [89] |
| Non-small cell lung cancer | Phase II | mDCs, autologous | Mild to severe AEs | TFF - 30% Sucrose cushion –TFF | 0.9% NaCl | −80 °C | [90] |

AE = adverse events, MSC = Mesenchymal Stromal Cell, imDC = immature Dendritic Cells, mDC = mature Dendritic Cells, UC = Ultracentrifugation, TFF = Tangential Flow Filtration.

There are currently at least eleven clinical trials in progress aiming to judge the safety and efficacy of different EV therapies. Since several potential concerns remain, continued research will be necessary to ensure that each specific application for EVs is safe. For example, almost all therapeutic EVs are produced from immortalized cell lines and as such may carry oncogenic material. Thus, it will be important to assess whether, and to what extent, repeated systemic EV treatments can induce cell transformation. Moreover, the safety of novel interaction surfaces, such as those of EVs from different cell sources, liposome-EV hybrids and surface conjugates, must each be tested individually. However, as EVs have been shown capable of replacing various methods of cell therapy and other dangerous interventions, consideration of their safety relative to current therapies must always be paramount. Despite these caveats, the work discussed above suggests a promising future for the safe clinical application of therapeutic EVs.

## 6. How: Cargo loading into EVs

In order to utilize EVs as drug carriers, a prerequisite is to find a strategy for efficient cargo loading. Two different approaches for EV loading can be distinguished: exogenous (i.e. after EV isolation) and endogenous loading (i.e. during EV biogenesis) [92]. Different techniques have been employed for exogenous loading of EVs, including electroporation [33], simple incubation [93,94], sonication [95], extrusion and freeze-thawing [94], with variable degrees of success. These loading techniques can, however, result in the aggregation of EVs or their cargo, and alter their physicochemical as well as morphological characteristics [96]. Uniquely, EVs can also be loaded endogenously, by exploiting the aforementioned sorting machinery of cells for the production and loading of biomolecules into vesicles. For example, cells can be loaded with specific cargo via direct transfection (e.g. for RNA therapeutics) [97] or co-incubation (e.g. for cytostatics) [98], after which EVs are loaded by the endogenous cellular machinery before their secretion into the extracellular space. Alternatively, in the case of RNA or protein drugs, cells can be engineered to stably express the therapeutic of interest, which can then be combined with approaches to increase active loading of the cargo molecule into EVs through fusion or interaction with molecules naturally enriched in EVs (Fig. 2, Table 2).

When natural vesicular trafficking mechanisms are well understood, these mechanisms may be exploited for the endogenous loading of exogenous proteins into EVs. For example, it has been reported that conserved late domain (L-domain) containing proteins are involved in MVB biogenesis, vesicular trafficking as well as EV loading. L-domain proteins aid in these processes primarily by recruiting ESCRT components and ubiquitin ligases [103]. One such L-domain-containing protein is Ndfip1, a ubiquitin ligase adaptor protein, which plays a role in protein trafficking on Rab5-containing early endosomes, and their packaging into EVs. One of its binding proteins is the Nedd4 family of ubiquitin ligases, which are known to be incorporated into EVs, as WW domains of Nedd4 proteins can interact with Ndfip1 via multiple PPxY L-domain motifs. This mechanism has been employed for the delivery of a model protein, Cre recombinase. Here, two WW domains were fused to Cre protein, resulting in its monoubiquitylation and packaging into EVs in a Ndfip1-dependent manner. As a result, a significant increase in Cre recombinase activity was found in recipient cells after addition of EVs derived from cells overexpressing WW-Cre and Ndfip1.

Similarly, RNA can be loaded into EVs through the engineering of cargo naturally found in EVs. For example, it has been shown that miR-199a can be loaded into EVs via the membrane protein Lamp2a. This was achieved by fusing Lamp2a to the HIV-1 Trans-Activator of Transcription (TAT), while modifying pre-miR-199a with the RNA TAT-cognate trans-activation response (TAR) peptide [99]. As a result, the modified miR-199a binds to TAT via the recognition of a unique site in the stem-loop structure of TAR. This approach resulted in a 65-fold increase of miRNA-199a-3p in isolated EVs. However, despite this enhancement in loading, addition of EVs to recipient cells did not lead to downregulation of miRNA-199a target genes. These findings are in line with a different study using a platform for endogenous loading of RNA loading called Targeted and Modular EV loading (TAMEL). This platform exploited the MS2 bacteriophage coat protein, which was introduced into EVs through fusion with an EV-associated protein (i.e. Lamp2b, Hspa8 or CD63), while the cargo mRNA molecule was engineered to contain a cognate MS2 stem loop [100]. Again, although this approach resulted in a substantial increase in EV loading, no mRNA translation was observed upon delivery to the recipient cells. These results may be attributed to inefficient endosomal escape for the specific EV types that were exploited in these studies, or to insufficient release of the cargo from the engineered loading platforms.

In an endeavour to achieve such release of cargo molecules from EVs to the cytosol of recipient cells, a reversible light-cleavable protein has been employed in an optogenetically engineered system, known as the «Exosomes for protein loading via optically reversible protein-protein interactions» module (EXPLORs) [101]. In this approach, a photoreceptor cytochrome 2 (CRY2) was fused to the cargo protein (e.g. Cre

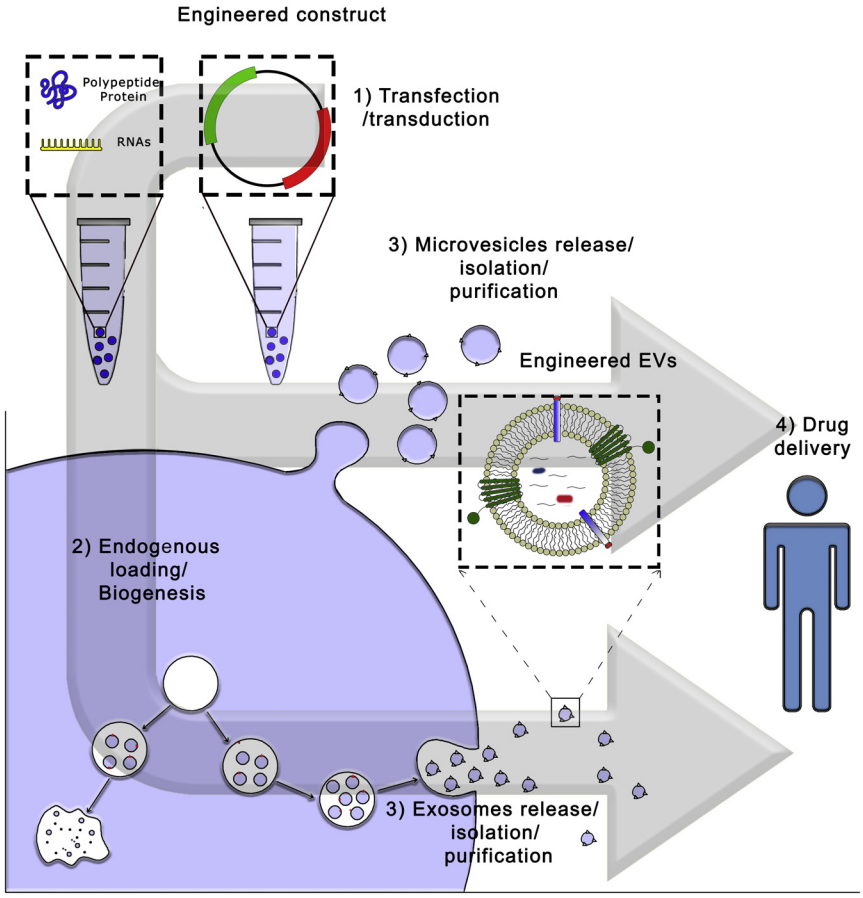


**Fig. 2.** A schematic diagram displaying the strategy for endogenous loading of engineered EVs. (1) Producer cells are engineered to express the desired cargo (e.g. RNA or protein) as well as other genes of interest (e.g. an RNA-binding protein). (2) During EV biogenesis, endogenous sorting of desired cargo takes place, which is followed by the release of the now loaded EVs (3). Engineered EVs are then isolated, purified and evaluated in cells/animals/patients (4).

recombinase) while CIBN, a shorter version of CRY-interacting basic-helix-loop-helix 1, was conjugated to the EV transmembrane protein CD9. In such a way, in the absence of blue light, engineered cargo is released into the intraluminal space of EVs. As a result, significant Cre activity was observed in the recipient cells upon the addition of these engineered EVs. Moreover, the same light-cleavable protein has also been adopted for the loading and delivery of a miR-21 sponge [102]. In this case, the CIBN was conjugated to a palmitoylation sequence that targets CIBN to the plasma membrane, whereas CRY2 was fused with an MS2 bacteriophage coat protein and the cargo miR-21 sponge was bound to the MS2 binding aptamer. Again, this reversible light-cleavable protein resulted in a significant increase of apoptosis in tumour cells as a result of functional delivery of the miR-21 sponge.

Another strategy for reversible cargo loading into EVs is using drug-inducible dimerization, one example of which is the association between the DmrA and DmrC domains. These are fragments of the FK506-binding protein (FKBP) and FKBP-rapamycin-binding protein (FRB), respectively, that only interact in the presence of a rapamycin analogue also known as the A/C heterodimerizer [105]. It has been shown that when membrane bound CherryPicker Red protein (a fusion protein composed of the fluorescent protein mCherry and the transferrin receptor membrane anchor domain) is fused to a DmrA domain and Cas9-sgRNA ribonucleoprotein (RNP) complex is linked to a DmrC domain, the two domains interact subsequent to the addition of the A/C heterodimerizer molecule. Subsequently, this promotes Cas9 RNP complexes to be packaged inside EVs, while dissociation of the complexes occurs after dilution of the A/C heterodimerizer once the EVs fuse with membranes inside the recipient cell [106]. Using this approach, EVs expressing vesicular stomatitis virus glycoprotein (VSV-G), termed gesicles, were utilized for the delivery of Cas9 RNP complexes targeting the Long Terminal Repeat (LTR) regions of integrated HIV provirus. Cells treated with these engineered gesicles displayed a reduction in the copy number of HIV provirus and the viral protein Nef.

As the biogenesis of specific populations of EVs is increasingly understood, it is now also becoming possible to specifically engineer and utilize these different populations. For example, it has been found that arrestin domain containing protein 1 (ARRDC1) induces the outward budding of ARRDC1-mediated microvesicles (ARMMS) from the plasma membrane, via recruitment of ESCRT-1 complex protein TSG101 [104]. As a result, an increase in the expression of ARRDC-1 results in an increase in ARMMs production. These features were exploited for the delivery of tumour suppressor p53 protein, by fusing ARRDC-1 directly to the N-terminus of a wild-type p53. Experiments showed that the functional delivery of p53 via ARMMs resulted in the expression of p53-dependent genes in recipient cells and promoted p53-dependent apoptosis in vitro and in multiple tissues in vivo. In addition, for the packaging and delivery of p53 mRNA, the aforementioned TAT-TAR system was employed, where TAT protein was fused to ARRDC-1 and the cargo mRNA was modified by adding the TAR RNA loop, which resulted in a significant increase in vesicular loading of p53 mRNA and in the expression of p53 target genes in A549 recipient cells. Successful delivery of sgRNA/Cas9 complexes was also achieved by fusing several WW-domains to Cas9, to which ARRDC-1 can specifically bind via its PPxY motifs. One hypothesis for the successful functional delivery of cargo

**Table 2**
An overview of different engineering approaches for endogenously loading cargo into EVs.

| Platform | Membrane protein | Fusion protein(s) | Cargo | Loading efficiency | In vitro effects | In vivo effects | References |
|---|---|---|---|---|---|---|---|
| Viral TAT/TAR interaction | Lamp2a | TAT-TAR | Pre-miR-199a | 65× enrichment, compared to EVs lacking TAT peptide | No change in mRNA expression in Sk-Hep-1 and Huh-7 was noted | – | [99] |
| MS2-bacteriophage coat protein (TAMEL) | Lamp2a, CD63, Hspa8, or Modified Lamp2b | MS2-MCP | mRNA | Enrichment strongly dependent on membrane protein | Cargo mRNA was not translated in PC-3 | – | [100] |
| Light-inducible loading system (EXPLOR) | CD9 | CIBN-CRY2 | Recombinant protein, or Bax and super-repressor IκB | 40× enrichment, compared to EVs loaded by extrusion | Reduction in tumour necrosis factor-α-induced translocation of the p65 subunit of NF-κB in HeLa cells | Induction of recombinant protein expression in heterozygous transgenic and wild-type mice | [101] |
| Light-inducible loading system with MS2-bacteriophage coat protein | Palmitoylation sequence | CIBN-CRY2 and MS2-MCP | miR-21 sponge | 14× enrichment, compared to EVs produced without blue light induction | Increase in the expression of PTEN proteins and apoptosis in K562 | – | [102] |
| L-domain containing protein | Ndfip1 | WW-tag | Cre protein | Not reported | Significant Cre recombinase activity was recorded in MEF, derived from mT/mG | An increase in number of recombined cells in multiple brain regions in Ai14 mice | [103] |
| Viral TAT/TAR interaction and L-domain containing protein | ARRDC-1 | TAT-TAR, WW-tag, or direct fusion | Wild-type P53, mRNA p53, or sgRNA/Cas9 | ~540 cargo protein molecules per ARMM vesicle | ARRDC1-p53-containing ARMMs induced transcription of *MDM2* and *p21* in H1299 cells. ARMMs containing TAR-p53 increased transcription of Mdm2 and p21 in A549 recipient cells. | ARRDC1-p53 ARMMs resulted in a significant induction of apoptosis post irradiation in both spleen and thymus *TP53* KO mice. | [104] |

molecules via the ARMMs platform is that ARMMs enter cells by direct cell fusion, bypassing the endolysosomal pathway and avoiding cargo degradation. This idea is supported by the fact that ARMMs share a similar biogenesis pathway to that of fusogenic viruses.

Overall, it is clear that there are various platforms and pathways that can be utilized for the endogenous loading of cargo molecules into EVs; however, it is important to note that loading efficiency and functional delivery are highly dependent on the platform as well as the choice of EV-associated molecules used for targeted loading, which must be optimized for each specific application. Moreover, whether different populations of EVs are equally equipped for functional uptake and cytosolic delivery of cargo molecules remains an important area of inquiry.

## 7. How: Functionalized EVs for targeted delivery

Besides EVs' unique possibilities for cargo loading, EVs may also offer beneficial features for drug delivery in terms of targeting. It has been established that EVs have intrinsic targeting properties, at least to some extent, as lipid composition and protein content can influence EVs tropism to specific organs [36]. For example, different types of integrins have the ability to alter the pharmacokinetics of EVs and increase their accumulation in brain, lungs, or liver, depending on the integrin type [107]. In addition, EVs containing Tspan8 in complex with integrin alpha4 were shown to be preferentially taken up by pancreatic cells [108]. Similarly, EV lipid composition can affect their uptake, as for example phosphatidylserine is known to be involved in the uptake of EVs by macrophages [109]. In addition, EVs can be further tailored for targeted delivery through engineering of producer cells via similar mechanisms as those described above for cargo loading.

Several efforts have already been made to achieve targeted delivery of EVs. For example, it has been demonstrated that addition of siRNA-carrying dendritic cell-derived EVs expressing Lamp2b, fused to either neuron-specific rabies viral glycoprotein (RVG) peptide or a muscle specific peptide, led to specific, targeted gene knockdown in neuronal cells (Neuro2A) or muscle cells (C2C12), respectively [33]. Similarly, a very recent study exploited EVs isolated from cardiosphere-derived cells (CDC) for targeted delivery by fusing the N-terminus of Lamp2b (exposed on the EV surface) to a cardiomyocyte specific peptide (CMP) [110]. EV uptake by primary neonatal mouse cardiomyocytes was increased approximately 18-fold for CMP-targeted EVs, as compared to unmodified EVs. Various other cell lines were explored to verify uptake specificity which did not show a significant difference between CMP-targeted and unmodified EVs, confirming peptide specificity towards cardiomyocytes. Moreover, in vivo studies showed a significant enhancement in retention of CMP-targeted EVs in the heart as compared to unmodified EVs after intramyocardial injection. Lamp2b fusion proteins have also been used to target EVs to tumour tissue. EVs derived from immature dendritic cells (imDCs) were engineered to express Lamp2b fused to iRGD peptide, which is well-known to target αvβ3 integrin that is characteristically overexpressed by angiogenic vasculature [111]. Isolated iRGD EVs were loaded with the chemotherapeutic drug Doxorubicin (Dox) by electroporation, after which EVs were injected into MDA-MB-231 tumour-bearing mice. A marked increase in EV accumulation in tumours, as well as suppression of tumour growth, was found in mice treated with iRGD-targeted EVs as compared to mice treated with untargeted EVs.

Despite these encouraging results, concerns have been raised regarding the efficiency of peptide display on EVs during their biogenesis, as engineered EV surface proteins may be degraded intracellularly, for example by endosomal proteases [112]. Indeed, peptides bound to the N-terminus of Lamp2b have been shown to be degraded in endosomes during EV biogenesis through acid-dependent proteolysis. For the purpose of protecting such peptides from proteolytic degradation, GNSTM glycosylation motifs can be included in the peptide-Lamp2b fusion protein, which protect the peptide from degradation [112]. Alternatively, to overcome the aforementioned problems that might result in peptide degradation, targeting peptides can be directly tethered to the plasma membrane. For example, HEK293 cells have been engineered to express the EGFR-targeting GE11 peptide fused to the transmembrane domain of platelet-derived growth factor receptor [113]. Upon intravenous injection of EVs carrying GE11 on their surface into mice bearing EGFR-positive tumours, a pronounced increase in tumour accumulation was recorded. An

alternative approach is based on the glycosylphosphatidylinositol (GPI)-anchored protein decay-accelerating factor (DAF), which is known to be selectively released in EVs during the reticulocyte maturation process [114]. When DAF-derived GPI-anchor signal peptides were fused to anti-EGFR nanobodies, known as EGa1, these nanobodies were highly enriched on the surface of EVs following the cleavage of DAF peptide by GPI transamidase enzymes during post translational modification. As a result, a drastic increase in EV binding to cells overexpressing EGFR under both static and dynamic conditions was observed.

Taken together, conjugating targeting moieties onto the surface of EVs by using the endogenous cellular machinery shows promise in improving the delivery of EVs to their desired site of action. This may help to enhance therapeutic efficiency, while abrogating off-target side effects of encapsulated therapeutic cargo.

## 8. How: Upscaling, isolation, storage and GMP production

The endeavour to bring EV therapeutics into clinical trials and to industrial scale production will require upscaling of cell culture conditions, isolation and purification methods. Moreover, storage conditions that maintain EV functionality must be used, while the entire production needs to adhere to current Good Manufacturing Practices (cGMP). Upscaling EV production to industrial levels is still in its infancy and it is important to decide early on on approaches capable of producing sufficient quantities of EVs for the intended clinical development programmes and subsequent in-market supply. So far, the upscaling efforts for EV production have focused on a 'scale-out' approach at small to medium scale, with large numbers of culture flasks, cell factories or medium-sized bioreactors, all of which produce up to 8-10 L of conditioned medium [84–86,88–90,115–117]. Scaling up cell culture procedures needs to be assessed for each product, based on the producer cell culture method (e.g. adherent vs. suspension), amount of EVs required (e.g. for local vs. systemic administration), quantity of EVs produced by the cell of choice, and whether the patients will require single or repeated injections. Two recent studies have shown that MSC and cardiac progenitor cell (CPC) derived EVs can be obtained in clinically relevant amounts from either bioreactor or HyperStack systems, respectively [116,117]. Both studies indicated that neither the cells nor the EVs change phenotype during the scale-up of the cell culture procedure. While scale-up into HyperStack systems and bioreactors can meet short term clinical needs, further work is required to establish large scale cell culture for commercialisation and in-market supply.

The next step in the manufacturing process is the isolation of EVs in large-scale format. One important aspect to take into account during the planning of the isolation method is the required purity of the preparation. For certain applications, EV purity may not be vital and isolation of crude vesicular secretome fractions (VSFs) can be sufficient [118]. Scalable isolation methods are beginning to be developed, with tangential flow fractionation (TFF) commonly utilized as a first dia-filtration and concentration step [117,119]. Following TFF, the product is often further purified by ultracentrifugation (UC) or by other clean up steps, such as bead-elute chromatography [91,117,119]. While bead-elute chromatography is scalable, ultracentrifugation is difficult to scale up and problematic to scale-out. For the clinical trials that have been published to date, the most common purification method is TFF combined with UC protocols (see Table 1 for a list of completed clinical trials and the isolation method used) [70,86,88–90,115]. However, the above-mentioned purification methods rely on differences in size and/or density to separate the EVs from non-vesicular material, which makes it difficult to purify specific subpopulations, or a specific drug-loaded EV population. Other alternatives for large-scale purification include anion exchange liquid chromatography and immune capture approaches, such as affinity chromatography, which have the ability to purify subpopulations [120–122]. However, elution of intact EVs is a challenge for both methods. Very recently, a publication demonstrated EV capture using a CD63 aptamer and subsequent elution using a competing oligonucleotide [123]. This approach appears to be able to purify subpopulations and can be scaled similar to traditional immune capture approaches. One advantage for all capture techniques is the possibility, in theory, to purify only drug loaded or engineered vesicles. Finally, PEG-precipitation has been employed in clinical settings for MSC EV purification [84]. Albeit a crude method that merely precipitates EVs rather than purifying them, the precipitation method may still be a viable option for large-scale production. Since the most critical issues for large-scale purification intended for clinical trials or industrial production are the need for demonstrated safety of the drug product and the need for consistent manufacture between batches, these can be considered more important than absolute purity.

After the purification of EVs at large scale, the EV product needs to be stored in a suitable container-closure system, at a temperature and in a storage buffer that maintain EV stability. Storage of the EV product is often an overlooked area, which requires more attention. Current consensus seems to support storage of EVs at −80 °C [124], however, there are few studies that have investigated the impact of storage buffer. Another important aspect is the storage container, since certain surfaces can bind to and alter the characteristics of EVs [125]. Hence, which plastics and coatings to use in the storage container must be carefully considered before storage. Cryopreservation with cryoprotectants (CPAs) is commonly used to decrease osmotic damage and increase the stability of proteins and cells during freezing. This is commonly achieved through increasing viscosity and affecting ice-formation during freezing. In previous clinical trials, vesicles were stored in either PBS, 0.9% sodium chloride, cell culture medium M199 + 25 mM Hepes or 20 mM Tris/1 mM $MgCl_2$/5% sucrose/100 μg/mL human serum albumin (see Table 1 for storage conditions used in EV clinical trials) [70,84,85] [86,88–90,115]. Another storage solution proposed for clinical grade EVs is Plasma-Lyte A, which is an isotonic solution with a similar salt composition to plasma, however without the presence of proteins or sugars [116–118]. Although only few studies have systematically compared different EV storage formulations, it is increasingly evident that CPAs do increase the stability and functionality of EVs after storage at low temperatures. For example, Trehalose is an FDA approved excipient for several protein and cell therapies and has been shown to increase the stability of EVs [126,127]. Another storage alternative is lyophilisation or freeze drying of EVs, which has been shown to confer increased stability and shelf life on EVs in several studies [126,128]. For any EV translational research, it will be important to examine different storage conditions and evaluate their effects on functional assays in order to find an optimal storage condition. However, EVs from different cell sources and/or EV subpopulations may have different optimal storage conditions and thus the storage conditions may have to be optimized for each novel EV-based therapeutic.

Lastly, all the above steps need to be cGMP compliant, with manufacturing personnel following cGMP protocols. In order to prevent cross-contamination, workflows should ideally be operated as a closed system. When closed operation is not possible, any open unit operations would need to be operated in biological safety cabinets or isolators. Nevertheless, two recent published articles regarding production of MSC- and CPC- derived EVs under GMP conditions did not use a closed system during the entire workflow [116,117]. It is noteworthy that one of the studies did include electroporation-based loading of siRNA into the GMP workflow, highlighting that it is possible to implement exogenous cargo loading of EVs in a GMP compliant manner [116]. Parenteral drug products are required to be sterile requiring a sterilisation technique that is compatible with the drug product. For therapeutic EVs, especially where RNA species are important, radiation or chemical virus/bacteria inactivation will probably impact therapeutic efficiency and thus appear to be inappropriate. An alternative is 0.2 μm sterile filtration, which can be implemented in most EV purification workflows before storage. However, if therapeutically active EVs are larger than 200 nm this method is obviously not feasible.

Over the last few years, the EV field has matured towards clinical translation, and while some questions regarding isolation, storage and GMP production have been solved, many challenges remain. For example, straightforward large-scale production, yielding EVs with good purity, has so far been difficult to achieve. Moreover, optimal storage conditions of therapeutically relevant EVs have not been thoroughly evaluated.

## 9. Conclusion and future perspective

Due to their natural involvement in the intercellular exchange of biomolecules, EVs hold great potential as a novel drug delivery vehicle, especially for delivery of biotherapeutics, which may be loaded into EVs using the endogenous cellular EV packaging machinery. EVs possess distinctive characteristics that favour their utilization as a drug delivery system over synthetic ones. These include their ability to cross physical barriers, their inherent targeting characteristics, their ability to exploit natural intracellular trafficking pathways, as well as their increased biocompatibility. By engineering cells from which EVs are derived, various platforms for loading EVs and conjugating targeting moieties to them have been developed. These have resulted in highly encouraging proof-of-concept studies in preclinical models. Nonetheless, more effort needs to be made to achieve translational applications of EVs. Obstacles that need to be overcome towards clinical utilization include upscaling of the EV production and isolation process, as well as guidelines for appropriate storage. Moreover, increasingly in-depth investigations into EV biogenesis, cargo sorting, EV subpopulations, and internalization and trafficking pathways in recipient cells are crucial in order to gain more insight into strategies to further improve EVs as drug carriers.

## Declaration of Competing Interest

J.Z.N. is consultant for and has equity interests in Evox Therapeutics Ltd. R.M.S. is CSO of Excytex bv. S.EL.A. is co-founder and shareholder in Evox Therapeutics. P.V. serves on the scientific advisory board of Evox Therapeutics.

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