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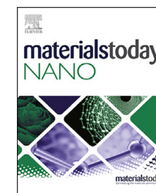

Converting extracellular vesicles into nanomedicine: loading and unloading of cargo

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Extracellular vesicles (EVs) are membranous containers that are secreted by multiple cell types and actively transport biomolecules such as lipids, proteins, and nucleic acids to distant cells, thereby inflicting phenotypic changes. In addition to their use in disease diagnosis, EVs have emerged as powerful tools for disease treatment. Specifically, the natural transport capacity of EVs can be exploited for drug delivery purposes. In this review, we focus on the key technologies that are used to 'design' EVs for their use as biological delivery vehicles. We provide a comprehensive overview of (i) methods for the loading of EVs with therapeutic cargo, (ii) methods for EV surface functionalization to direct EVs to target cells, and (iii) methods to stimulate cargo release from EVs. Finally, we discuss the remaining and upcoming challenges for the clinical translation of EV-mediated drug delivery.

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1. Introduction

The performance of many pharmaceuticals in disease treatment is suboptimal because of poor bioavailability and toxicity, which prevents their administration at a therapeutic dose. Nanoparticles hold high potential as effective drug delivery systems by preventing drug delivery to non-target tissues, thus enhancing drug specificity and reducing toxicity. Ultimately, active targeting to a specific tissue site is the holy grail of drug delivery systems. To prevent fast clearance of nanoparticles from the blood by liver, spleen, and kidneys, the nanoparticles should be prevented from filtration, and recognition by phagocytic cells. Moreover, nanoparticles should refrain from stimulating an immune response, and inducing toxicity [1]. The coating of nanoparticles with a layer of polyethylene glycol (PEG) is widely used as a method for protection against removal by the reticuloendothelial system [2], although drawbacks such as hypersensitivity to and antibody formation against PEG have been documented [3–6]. The development of 'safe' nanoparticles is largely focused on the use of biodegradable and biocompatible materials [7–9].

Despite significant developments, drug delivery systems still face the big challenge of escaping from the host surveillance system, while efficiently delivering cargo at a specific location in the body without harming non-target tissues [1]. In this regard, extracellular vesicles (EVs), natural carriers secreted by a variety of cell types, have emerged as a powerful tool for drug delivery.

Once put away as 'garbage bags' and 'platelet dust', EVs are now known to function as messengers between cells [10,11]. Cellular information in the form of proteins, nucleic acids, and lipids can traffic between neighboring and distant cells via EVs (Table 1) [11]. EVs have been implicated in various developmental as well as pathogenic processes such as neural development [12,13], wound healing [14], cancer metastasis [15,16], immune response mediation [17], host–parasite interaction [18], and progression of neurodegenerative disease [12,19,20].

EVs are secreted extracellular structures that are enclosed by a lipid bilayer. They include microvesicles (MVs), exosomes, and apoptotic bodies (ApoBDs), and are categorized on the basis of their biogenesis pathway [44]. While MVs are formed by outward budding of the plasma membrane of cells, exosomes are formed by the inward budding of the limiting membrane of endosomes, thus forming multivesicular bodies (MVBs). Upon fusion of MVBs with the plasma membrane the vesicles are released in the extracellular

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Table 1
Principal components of EVs.

Class	
Targeting/adhesion molecules	Integrins (α4β1, αMβ2, β2, αLβ2), ICAM-1/CD54, MFG-E8/lactadherin [21–26]
Tetraspanins	CD9, CD37, CD53, CD63, CD81, CD82 [22,23,25,27–30]
Heat shock proteins	Hsc70, Hsp84/90 [22,28,31,32]
Antigen presentation proteins	MHC class I and II [23,27,28,32–35]
Cytoskeletal proteins	Tubulin, actin, cofilin, myosin [21,22,28,36]
Membrane transport and fusion proteins	Annexins (I, II, IV, V, VI), RAB proteins (RAB7, RAP1B, RABGDI) [21,36]
Signal transduction proteins	G-proteins (Gi2α), 14-3-3, protein kinases, flotillin-1 [36,37]
Enzymes	Peroxidases, pyruvate and lipid kinases, enolase-1, GAPDH [28,37]
Protein synthesis molecules	EEF1A1, EEF2, ADP ribosylation factor [37]
Transmembrane molecules	A33 antigen, P-selectin, cell surface peptidases (CD13, CD26), ATPase channels [28,29,37]
Antiapoptotic proteins	Alix, thioredoxin peroxidase [36,37]
MVB formation proteins	Tsg101, Alix, Clathrin [36,37]
Lipids	Phosphatidylserine, cholesterol, ceramide, sphingomyelin, lysophosphatidylcholine, arachidonic acid, prostaglandins, and leukotrienes [29,38–40]
Nucleic acids	DNA, RNA (mRNA, miRNA) [41–43],

ICAM, intercellular adhesion molecule; MFG-E8, milk fat globule EGF factor VIII protein; MHC, major histocompatibility complex; GAPDH, glyceraldehyde 3-phosphate dehydrogenase-activating protein; EEF, eukaryotic translation elongation factor; TSG101, tumor susceptibility gene 101.

space [45] (Fig. 1). MVs and exosomes show significant overlap in their (cytosol-derived) cargo, their biological functions, and underlying molecular mechanisms, including their cellular entry pathways [46]. The third type of EVs, ApoBDs, fall in the range from 50 to 5000 nm [47], and are generated from cells undergoing apoptosis, which is characterized by plasma membrane blebbing [10,48–50]. Owing to the vast increase in EV isolation methodologies (Table 2), other EV subpopulations have been discovered, including exomeres, small and large exosome-like vesicles, and mitochondrial protein-enriched EVs [50–53], while also raising the possibility that not all EV subpopulations are comprehensively defined. Currently, transmission electron microscopy, nanoparticle tracking analysis, and dynamic light scattering [54] are the mostly used techniques for particle size determination, while flow cytometry, western blotting, and mass spectrometry (MS) serve to identify the protein composition [55]. Additionally, the lipid and carbohydrate fingerprint can be obtained by lipidomic analysis [56,57] and lectin microarray technology [58], respectively. Generally, the required EV yield and purity, together with the ease and costs of the isolation procedure govern the choice for a specific EV isolation procedure and following characterization techniques [59].

In this review, we will focus on the two EV types that are most widely explored as drug delivery vehicles: exosomes and MVs, while collectively referring to them as EVs [50–52,67]. Specifically, we provide a detailed account of the available methods for the loading of EVs with (therapeutic) cargo, EV unloading mechanisms, and the challenges and prospects of the development of EVs as drug delivery vehicles. Of note, the third type of EVs, i.e., ApoBDs, has received less attention in drug delivery research thus far, mainly because of their broad size distribution and engulfment of large ApoBDs by phagocytes. However, the potential of ApoBDs as drug delivery vehicles is increasingly being recognized, especially that of small ApoBDs [68–70].

2. EVs as drug delivery systems

The potency of EVs as a new class of nanocarriers owes to their unique properties as information carriers, including their intrinsic homing ability, biocompatibility, cell-specific targeting, non-

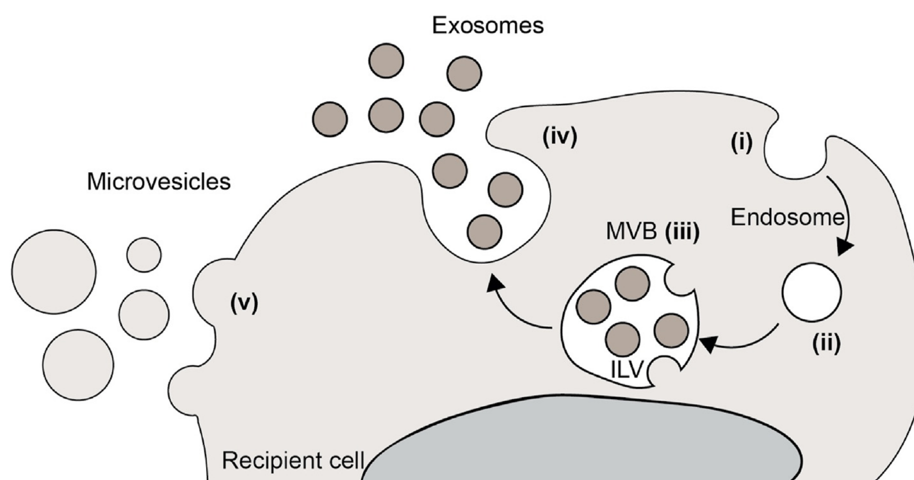

Fig. 1. Biogenesis of microvesicles and exosomes. Exosomes are endosome-derived vesicles. Plasma membrane invaginations (i) pinch off and form endosomes (ii) MVBs (iii) are formed when the limiting membrane of an endosome buds inwards and forms intraluminal vesicles (ILVs). Upon fusion of MVBs with the plasma membrane the ILVs, now called exosomes, are released in the extracellular space (iv). Microvesicles are plasma membrane–derived vesicles. They form when the plasma membrane buds outwards and undergoes fission (v).

Table 2
Exosome isolation methods including advantages and disadvantages.

EV isolation/purification method	Description	Advantages	Disadvantages	Reference
Differential ultracentrifugation with density gradient centrifugation	• Differential centrifugation of cell culture supernatant or body fluids at increasing speeds (300 g: removes cells; 1,000 g: removes cell debris; 10,000 g: removes MVs; 100,000 g: pellets exosomes) • Additional use of a sucrose density gradient during ultracentrifugation separates contaminants from EVs	• High EV yields • High EV recovery • High EV purity	• Time consuming (62–90 h) • Reduced protein and RNA recovery • Highly sensitive to various parameters, e.g., the applied speed (g-force), the centrifuge characteristics (rotor type, angle of rotor, radius of centrifugal force), the solution viscosity, and the operator variability	[60–62]
Filtration paired with centrifugation	• Size-based separation using ultrafiltration membranes with defined molecular weight or size exclusion limits	• Useful for isolation of EVs from large volumes (>1 L) of conditioned medium • Suitable for clinical applications • Selective isolation based on molecular weights	• Loss of EVs due to binding to the membranes • Remnant contamination go smaller components • Blockage of the membrane pores in concentrated fluids due to increased applied force	[62]
Immunoaffinity capture	• Trapping EVs on plates, filters, matrices, or beads bearing antibody against specific EV surface proteins • Most common targets are tetraspanins (CD63, CD9, CD81, etc.) and TSG101	• Simple and rapid • Useful for rough characterization of EVs • Does not require ultracentrifugation • Can be analyzed by flow cytometry using fluorophore-conjugated antibodies • High EV isolation specificity • Highly pure EV isolate	• Not suitable for large-scale EV purification • Possibility of loss of EV functionality during release from the captured matrix • Restricted to a subpopulation of marker-positive EVs • Dependent on appropriate selection of EV surface target and availability of the target antibody • Low yield	[24,60,62]
Size exclusion chromatography	• Separation of EVs based on differential hydrodynamic diameters by passing the heterogeneous EV solution through a column containing beads with pores of different sizes	• Clear separation of large molecules from small molecules • Preserves the integrity and biological activity of EVs being separated • Excellent reproducibility and sensitivity • High EV recovery	• Long run time • Limited scalability for high-throughput applications • Needs to be combined with another isolation technique to concentrate the final EV preparation • The use of an extra force to reduce the run time might cause the deformation and break-up of EVs	[60,62]
Polymer-based precipitation	• Polyethylene glycol–based precipitation of EVs, performed by addition of EV suspension to polyethylene glycol-containing solution and low speed centrifugation • The most commonly used commercial product is ExoQuick-TC from System Biosciences	• Easy to use • Does not require specialized equipment • It can be rapidly performed • Pre- and post-isolation steps can lead to a purer isolated EV fraction	• Co-isolation of non-vesicular contaminants such as lipoproteins and polymer residues	[62,63]
Microfluidic technologies	• Three main techniques founded on size (trapped in a porous-ciliated silicon microstructure), density (pressure or electrophoresis-driven membrane filtration with a specific pore size), and immunoaffinity (chip coated with antibody against EV surface protein) based EV isolation • Innovative techniques including size and density-dependent EV separation through ultrasound-operated acoustic nanofilter, electrophoretic or electromagnetic manipulation-driven separation	• Small volume of starting material is required; highly pure EV isolation; minimal processing time; high-throughput analysis of EV contents	• Low collection efficiency • Contamination with MVs of similar size • Not suitable for EV isolation from large samples	[64–66]

EV, extracellular vesicles; MVs, microvesicles; TSG101, tumor susceptibility gene 101.

immunogenicity, broad distribution in biological fluids, and easy penetration across physiological barriers [10,71]. In addition, the natural cargo of EVs may contain therapeutic molecules, which can be harnessed for therapeutic interventions [72]. Despite that the role of EVs in natural cargo transfer is being questioned, their potency as nanocarriers with accompanying advantages as non-immunogenicity, remains worthwhile to explore [73–75]. To exploit EV drug delivery potential, methods have been developed to introduce cargo of exogenous origin into EVs as well as to maximize their efficacy of targeting and delivery. Here, we discuss the strategies hitherto employed for EV cargo loading, targeting, and cargo unloading.

2.1. Loading cargoes

There are three general approaches to generate EVs that are loaded with therapeutic substances (Fig. 2): (1) exogenous EV loading: loading of cargo into pre-formed EVs, i.e., post-isolation; (2) endogenous EV loading: cargo loading during EV biogenesis, i.e., through engineering of cells prior to EV isolation; and (3) *in vivo* production of loaded EVs through implantation of genetically engineered cells or *in vivo* transduction/transfection of cells. Although each method has its advantages, the overall outcome of each technique is primarily influenced by the nature and type of the cargo molecule. Therefore, in this section, we will focus on different biomolecule species and the methodologies employed for their loading into EVs, both endogenously and exogenously (Figs. 3 and 4). An overview of the loading methods per molecular species is provided in Table 3. For a discussion of the advantages and disadvantages of the methods we refer the reader to Refs. [76–79].

2.1.1. siRNA

siRNAs are small non-coding RNA molecules that, upon their intracellular delivery, mediate the degradation of complementary mRNA and thereby silence gene expression. However, they are rapidly degraded in circulation and poorly enter cells due to their hydrophilic nature, hence their delivery is mediated by nanoparticles, including EVs [172–176]. Importantly, siRNA molecules are easily loaded into EVs owing to their small size (20–25 nucleotides) and are the most studied molecule for EV-mediated delivery.

Alvarez-Erviti et al. loaded EVs with siRNA against BACE1 (beta-secretase 1), an enzyme relevant to Alzheimer's disease, by means of electroporation (Fig. 3-1). In electroporation, short high-voltage pulses are used to produce transient pores in (cell) membranes, which allow for the passage of small molecules [177]. Intravenous injection of the BACE1 siRNA-loaded EVs into wild-type mice, led to successful BACE1 knockdown [80]. However, a follow-up study reported that electroporation induced siRNA aggregate formation, which caused an overestimation of the loading efficiency into EVs [178]. Nevertheless, electroporation still remains a popular method for siRNA loading, largely due to the realization of the desired therapeutic effect. For example, reduction of alpha-synuclein aggregates in the brain [81], alleviation of inflammation by reducing the expression of ICAM-1 (intercellular adhesion molecule 1) in microvascular endothelial cells [82], and reduction of oncogenic RAS expression and cancer suppression in multiple mouse models [83] have been obtained with EVs that were loaded with siRNA by electroporation.

Recently, as an alternative to electroporation, a modified calcium chloride–mediated 'transfection' method (Fig. 3-2), previously developed for loading microRNAs (miRNAs) into EVs [84],

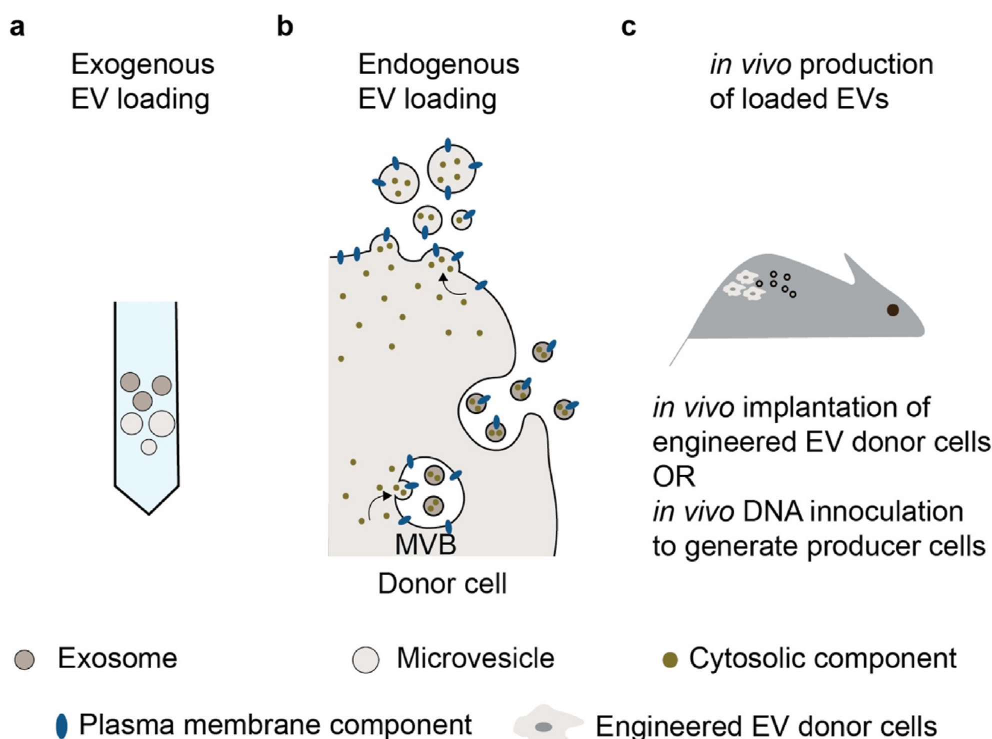

Fig. 2. EV loading approaches. Therapeutic biomolecules including RNA, DNA, protein, lipids, and small-molecule drugs can be loaded into EVs via exogenous incorporation (A, post-EV isolation) or endogenous incorporation during biogenesis of EVs (B, pre-EV isolation). The third approach involves *in vivo* generation of designer EVs by implantation of (*ex vivo*) engineered EV donor cells or *in vivo* DNA inoculation to generate producer cells (C).

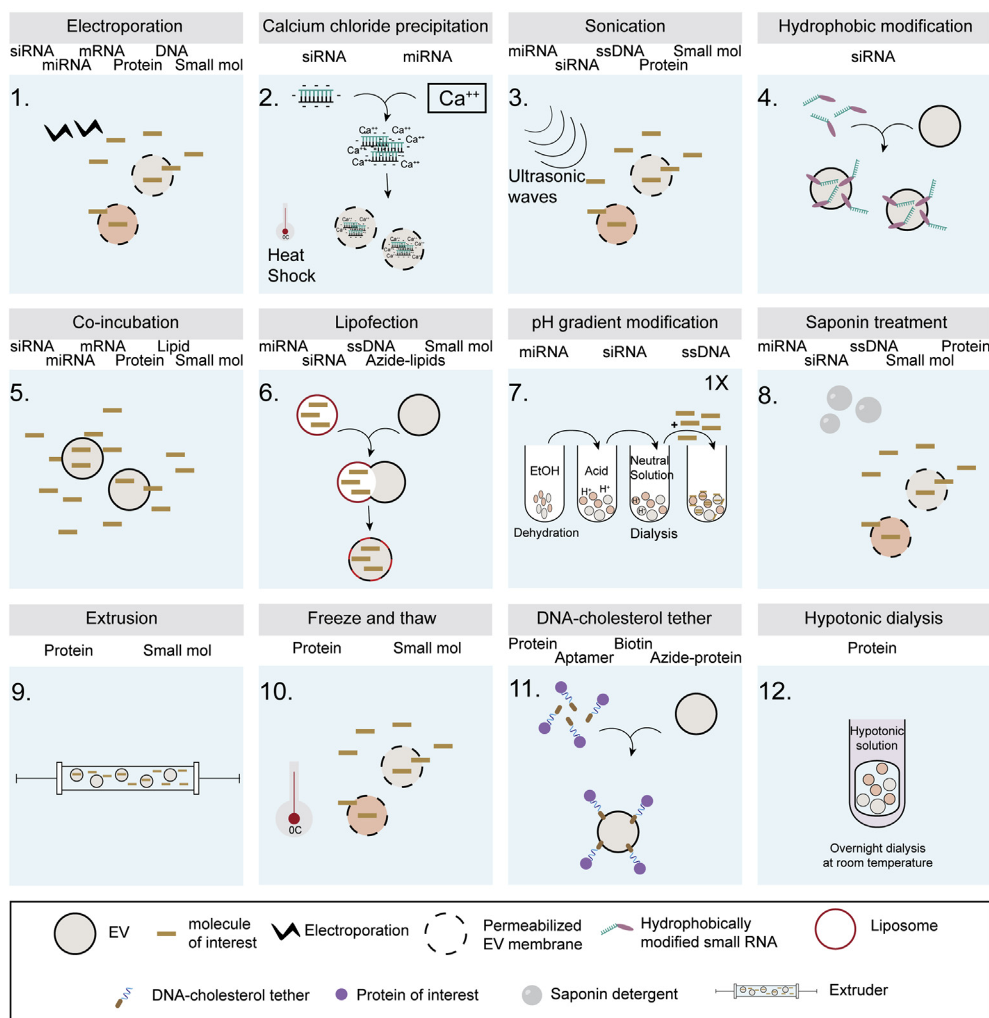

Fig. 3. Exogenous EV loading strategies. 1. Electroporation-induced membrane permeability enables loading of EVs with therapeutic molecules. 2. Calcium chloride–mediated precipitation of small RNAs followed by heat shock in the presence of EVs results in RNA encapsulation. 3. Momentary permeabilization of EV membranes by ultrasonic waves facilitates capture of therapeutics in EVs. 4. Hydrophobically modified small RNA molecules get inserted into the lipid bilayer of the EV membrane. 5. Simple co-incubation of EVs with therapeutics leads to their association. 6. Lipoplexes merge with the EV lipid bilayer, resulting in hybrid EVs containing EV and lipoplex lipids distributed in the membrane. 7. Subsequent dehydration, rehydration in acid, and neutralization facilitates entrapment of therapeutic biomolecules in EVs. 8. The mild detergent saponin solubilizes the EV membranes, aiding loading of therapeutics. 9. Extrusion of a dispersion of EVs and therapeutic molecules through a porous membrane leads to the incorporation of molecules in EVs. 10. Rapid freeze-thaw cycles lead to EV membrane disruption, which assists the loading of EVs with molecules. 11. A DNA-cholesterol tether is inserted into the EV membrane via the hydrophobic domain, while DNA acts as a conjugation entity for the attachment of various biomolecules. 12. Dialysis of EVs against a hypotonic solution that contains therapeutic molecules results in the loading of molecules in EVs.

was used for loading EVs with siRNAs. In this transfection method, calcium ions form microprecipitates with negatively charged chloride ions and nucleic acids. A subsequent heat shock triggers transient pore formation in the EV membranes, facilitating incorporation of nucleic acids into EVs [179]. EVs loaded with siMyd88 (an miRNA mimic) beneficially modulated lung inflammation *in vivo* [85]. Importantly, this method was compared with electroporation in the same experimental setting and was found comparable in terms of efficiency and convenience.

Sonication (Fig. 3-3) is an emerging alternative to electroporation. In this method, EVs are mixed with nucleic acid cargo and sonicated with a probe sonicator. During this process the EV membranes are ruptured and reformed, facilitating the encapsulation of nucleic acids within the EVs [180–182]. Lamichhane et al. [86] showed that siRNAs against oncogenic HER2 (human epidermal growth factor receptor 2) could be efficiently loaded into EVs by sonication for use in an animal model of breast cancer. Importantly, less large siRNA aggregates (>300 kDa) were formed during sonication compared to electroporation, and higher cellular uptake of EV-associated siRNA was observed. On the con side, a limited functional effect, i.e., limited siRNA-mediated gene silencing, was observed. Similar effects were seen for EV-mediated miRNA and ssDNA delivery in the same study. Sonication is an easily scalable strategy and has the capacity to load EVs with relatively bulky molecules. However, this may cause a significant change in the EV size [87,88], which may compromise EV function, specifically drug delivery performance. Thus, this method needs further optimization and development.

Recently, in order to increase the extent and ease of siRNA loading and delivery, siRNAs were modified with TEG (triethylene glycol)–cholesterol moiety to generate hydrophobically modified siRNAs (hsiRNA) for easy incorporation into the EV lipid bilayer

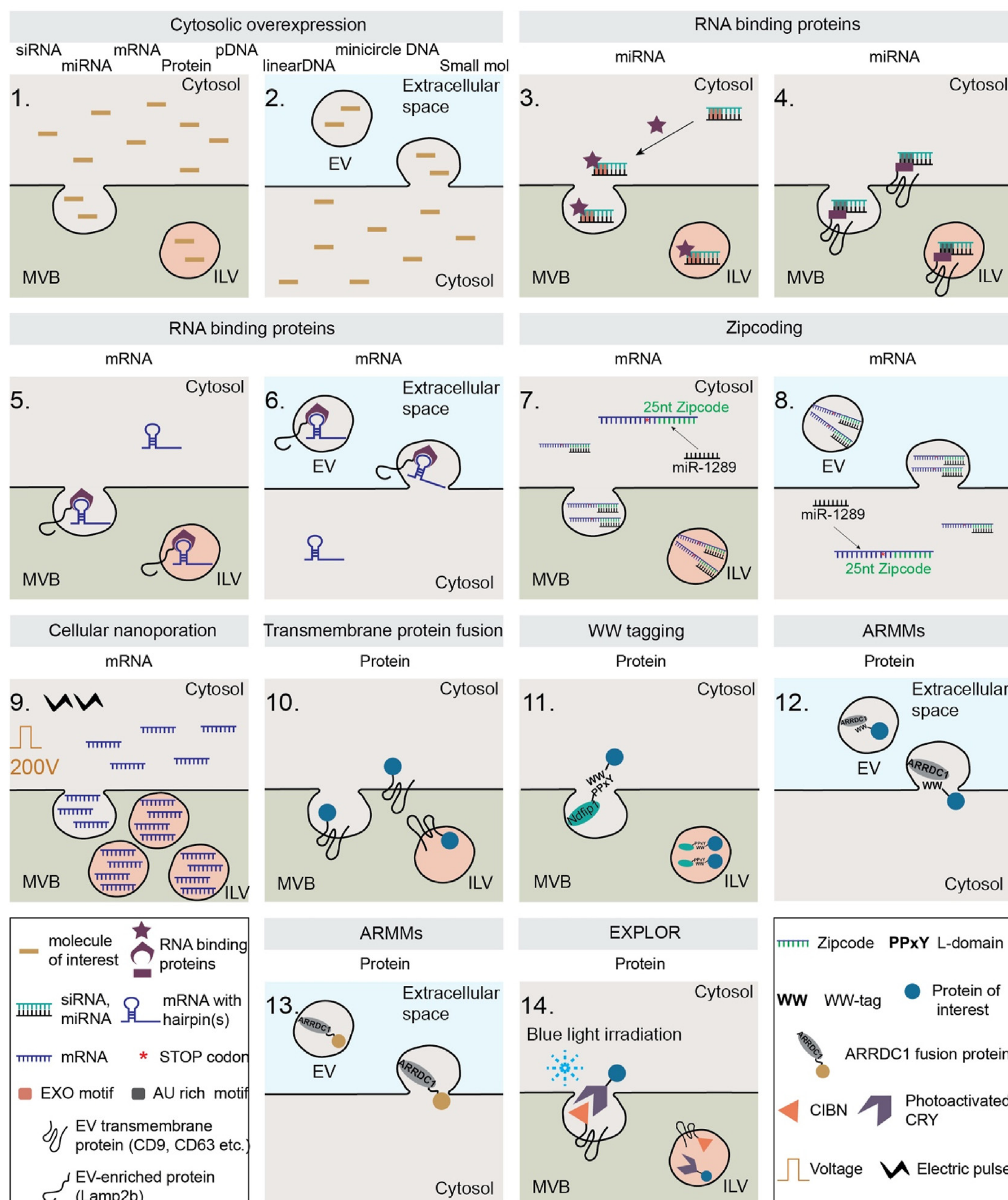

Fig. 4. Endogenous EV loading strategies. 1 and 2. Cytosolic overexpression of a therapeutic entity drives its loading into EVs by mass-action effect. 3 and 4. EV-enriched proteins, from cytosol or membrane-bound, that contain an RNA binding domain recognize a specific motif in miRNAs. 5 and 6. Expression of a fusion protein between an EV-enriched protein and an RNA-binding protein mediates mRNA loading into EVs through recognition of a hairpin motif in mRNAs. 7 and 8. A 25 nucleotide zipcode sequence marks mRNA for miR-1289 recognition, and consequent enrichment in EVs. 9. Cells with pDNA in their extracellular environment are pulsed with focal and transient electrical pulses, resulting in pDNA shuttling into the cytosol and subsequent enhanced mRNA expression and MVB formation, resulting in mRNA loading into ILVs. 10. Expression of a fusion protein between an ILV-enriched protein and cargo protein ensures cargo loading into ILVs. 11. Interaction between the L-domain of an ILV marker protein and a WW-domain in cargo protein, mediates cargo loading into ILVs. 12 and 13. ARRDC1 interacting with a WW-tagged cargo protein or fused to a cargo protein mediates cargo loading into MVs. 14. Photoactivated protein–protein interaction between CRY fused to a cargo protein and CIBN connected to an ILV-enriched protein, mediates cargo loading into ILVs.

(Fig. 3-4). *In vitro* and *in vivo* applications of EVs loaded with TEG–cholesterol siRNAs resulted in a.o. efficient reduction in Huntingtin mRNA levels [89] and silencing of the expression of human antigen R (HuR) [90]. The loading of EVs with hsiRNAs was further improved by assessing the ability of various lipid conjugates to enhance siRNA loading and silencing efficiency. Of all the tested fatty acids, sterols, and vitamin conjugates, vitamin E was observed to facilitate maximum siRNA loading and functional effect [91]. In addition, loading parameters such as incubation time, volume, temperature, and EV-to-siRNA ratio were shown to influence loading and delivery efficiency. Although the simple incubation of EVs with hsiRNAs is able to overcome the challenge of electroporation-induced siRNA aggregation, a slight increase in the EV size was noted, suggesting siRNA deposition on the EV surface, which could render the EVs immunostimulatory [89]. Furthermore, chemical modification of cholesterol-conjugated siRNAs is required

Table 3
EV-loading methods for different molecular species.

Molecular species	Loading methods and referred literature
siRNA	Electroporation [80–83] Calcium-mediated transfection [84,85] Sonication [86–88] Hydrophobic siRNA [89–92] Simple incubation [93] Lipid-based transfection [94–97] Cytosolic (over)expression [98,99]
miRNA	Lipid-based transfection [100] Cytosolic (over)expression [101–106] RNA binding protein-mediated loading [107–110] Electroporation [79,111–118] Calcium chloride method [84] pH gradient modification [119]
mRNA	Cytosolic (over)expression [120–122] Electroporation [123] RNA binding protein-mediated loading [124,125] Cellular nanoporation [126]
DNA	Transfection of producer cells [106,127] Electroporation [128,129]
Protein	Electroporation [130] Saponin treatment [87,88] Extrusion [87,88] Rapid freeze (−80°C)/thaw (RT) cycles [87,88] Sonication [87,88] DNA-cholesterol tether [131–133] Cytosolic (over)expression of cargo [106,122,134–136] Cargo-EV marker protein fusion [137] Protein-protein interactions (with WW-tag [138], ARRDC1 mediated [139], EXPLORs [140])
Lipids	Fusion with liposomes as induced by repeated freeze-thaw cycles [141] Co-incubation [142]
Small molecules	Co-incubation [143–154] Electroporation [155–161] Sonication [159,160,162] Cellular loading [163,163–169] Fusion with liposome-containing drugs [170,171] Incubation [159,160] Extrusion [159–161] Freeze-and-thaw [159,160] Saponin-assisted [161] Hypotonic dialysis [161]

to prevent degradation in the presence of EVs due to endogenous RNase activity [92]. However, the conjugation of a hydrophobic moiety to siRNAs for their loading into EVs may be unnecessary, because simple co-incubation of siRNA against phosphatase and tensin homolog (PTEN) with EVs (Fig. 3-5) was recently shown to result in 34% loading efficiency [93]. The intranasal administration of these EVs to rats with spinal cord injury substantially attenuated PTEN expression in the injured region leading to enhanced axonal growth while also improving neurovascularization and reducing micro- and astrogliosis. More importantly, significant functional recovery was obtained in rats following EV treatment. Direct comparison of cargo loading efficiency, potential immunostimulation, efficiency of cell entry, cargo unloading efficiency, and functional effect of EVs loaded with siRNA by the different methods is needed to decide on the method of choice.

Alternatively, lipid-based transfection reagents have been used for exogenous siRNA loading of EVs (Fig. 3-6). Using high-performance transfection reagent, EVs were 'transfected' with siRNAs for silencing mitogen-activated protein kinase-1 in human blood cells [94]. Similarly, Lipofectamine 2000 (lipofection reagent) was used to load EVs with antivascular endothelial growth factor siRNA for the treatment of brain cancer [95], and Exo-Fect (exosome transfection reagent) was used to load siRNA against lincRNA-cox2 to inhibit microglial proliferation *in vitro* and *in vivo* [96]. However, complex formation between the transfection reagent and siRNA may have occurred in addition to EV–siRNA complex formation, making it impossible to assign the observed functional effects to EV-mediated siRNA delivery [94,97].

In addition to the post-loading of EVs as discussed above, EVs can be pre-loaded with siRNA, i.e., prior to EV isolation. Pre-loading or endogenous loading can be achieved through the cytosolic expression of siRNAs in EV-producing cells (Fig. 4-1 and -2). For example, EVs isolated from cells overexpressing siRNAs against opioid receptor mu significantly reduced levels of mu mRNA and protein in the brain and inhibited morphine relapse in mice [98]. Similarly, EVs pre-loaded with siRNA against TGF-β1 (transforming growth factor beta 1) receptor suppressed TGF-β1 expression and signaling in tumor cells resulting in the inhibition of tumor growth and metastases [99]. Significant knock down of the BCR-ABL receptor, involved in chemotherapy resistance, was obtained with EVs pre-loaded with siRNA against the BCR-ABL receptor [183]. Although this EV loading method is broadly applicable, the RNA loading into EVs is proportionate to the cytosolic concentration of the RNAs and highly dependent on the identity of the parental cell type. In order to effectively increase EV pre-loading efficiency, very high siRNA overexpression is needed which may have undesired effects on the expression of other molecules in the parental cells, which may be carried forward to target cells via EVs [184].

2.1.2. miRNA

miRNAs are a natural component of EVs. Stoichiometric analysis of the miRNA content in EVs has revealed the presence of less than

one miRNA copy per EV [73,185–188]. Similar to siRNAs, miRNAs are small (18–25 nucleotides) RNA molecules that function in gene silencing [189] and can be loaded into EVs via pre- and post-loading methods. For example, human peripheral blood–derived EVs were post-loaded with miR-21 mimics or inhibitors using Exo-Fect, in a study on cardiac fibrosis [100]. The miR-21-loaded EVs successfully regulated target mRNA and protein levels in cardiomyocytes *in vitro* and *in vivo*. In a leading study by Ohno et al. [101], synthetic Let-7a miRNA, a tumor suppressor, was introduced in producer cells by lipofection and consequently pre-loaded into EVs by mass-action driving force. Furthermore, EVs isolated from miRNA-146a-transfected dendritic cells reduced autoimmunity in a neurological autoimmune disease model for myasthenia gravis, although this effect was attributed to a decrease in CD80/86 at the EV surface and not to EV-mediated miRNA delivery [102]. Similarly, two other studies [103,104] used EVs from miR150-5p and miR-124 transfected mesenchymal stem cells as a therapeutic intervention in *in vitro* and *in vivo* rheumatoid arthritis models and *in vitro* glioblastoma multiforme models, respectively. Finally, muscular atrophy and kidney fibrosis were reduced by injecting EVs loaded with miR-29, by viral transduction of the producer cells with an expression plasmid, into the muscles of mice undergoing muscular atrophy due to unilateral ureteral obstruction [105]. Of note, EVs that are loaded with miRNA through the transient transfection/transduction of producer cells with miRNA-encoding plasmid DNA (pDNA), may contain pDNA, which may be responsible for the phenotypic changes in recipient cells [106].

It has been shown that certain RNA-binding proteins mediate active sorting of miRNAs into exosomes through the recognition of sequence motifs in miRNA [190–194]. Overexpression of such RNA-binding proteins in EV-producer cells or introducing the sequence motifs into miRNAs, leads to active loading of the miRNA into exosomes [190]. Villarroya-Beltri et al. [190] identified two sequence motifs, EXOmotifs, for directing miRNAs into EVs and showed that sumoylation of the heterogeneous ribonucleoprotein A2B1 (hnRNPA2B1) RNA binding protein was required for its interaction with miRNA and its sorting into exosomes (Fig. 4-3). Wang et al. [107] loaded exosomes with miRNA (miR-104) against ICP4 (infected cell protein 4), a major regulatory protein of herpes simplex virus 1, through the introduction of a 21-nt RNA transfer sequence in miR-104 [108] The miR-104 exosomes effectively reduced ICP4 expression and, as a result, reduced infectious virus production from host cells.

Alternatively, RNA binding proteins can be exploited for miRNA loading by their fusion to EV membrane proteins (Fig. 4-4). In this regard, HuR, an RNA binding protein, was shown to specifically interact with AU-rich elements in RNAs [109]. Li et al. simultaneously overexpressed HuR fused to CD9, a protein abundantly present in the EV membrane, and miR-155, which contains AU-rich elements, in EV producer cells. The resulting EVs were highly enriched in functional miR-155 and efficiently reduced the target mRNA expression *in vitro* and *in vivo* [110]. Cargo loading of EVs prior to isolation through genetic engineering of parental cells guarantees proper encapsulation and protection of RNA molecules within the EV lumen, in contrast to the post-loading method which may result in localization of RNA at the EV surface. However, it is difficult to monitor the cargo-loading efficiency in pre-loaded EVs, and additional changes in EV composition that may arise from the overexpression of recombinant proteins in cells cannot be excluded. Similarly, damage to EVs during post-loading, e.g., by electroporation or sonication, cannot be excluded. Therefore, the preferred method for miRNA loading of EVs may depend on the research goals of the experimenter. Currently, electroporation is the most widely used method to load miRNAs into EVs, as reviewed in Refs. [79,111], probably because of its ease and speed.

In an early report [112], miRNA-155 was loaded into EVs from B-cells using an optimized electroporation protocol. These EVs successfully introduced the miRNA-155 into primary mouse hepatocytes and miRNA-155 knockout mice. Although this study showed successful delivery of miRNA into cells, functional outcome was not studied. Since then, many studies have used electroporation to load EVs with miRNAs to bring about phenotypic modulation *in vitro* and/or *in vivo*, such as miR-26a-loaded EVs [113] for inhibition of growth in cancer cells, miR-124-loaded EVs [114] for cortical neurogenesis in ischemia, miR-132-loaded EVs [115] to improve angiogenesis in ischemic diseases, let-7a-loaded EVs [116] for inhibition of cell proliferation in cancer cells, and miR-155-loaded EVs [117] for dendritic cell maturation. More recently, molecular beacons (MBs) and modified miRNAs (labeled with fluorophore–quencher pairs) loaded into EVs via simple co-incubation and electroporation, respectively, have been used to study the mechanism of EV-mediated miRNA release in recipient cells [195] and intracellular miRNA processing, specifically dicer detection [118]. MBs are hairpin-shaped DNAs with a stem-and-loop structure that contain a fluorophore and quencher pair in the stem. The MB becomes fluorescent when the loop binds to its complementary sequence, which causes the separation of the fluorophore–quencher pair in the stem [196,197]. Thus, only in the presence of a target sequence, the MB becomes fluorescent. This principle was used to visualize miRNA release in cells [195]: EVs containing miR-21 and miR-31 were loaded with MBs targeted against these miRNAs. Due to MB-targeted hybridization these EVs were fluorescent. Upon incubation of MB-EVs with cells, their colocalization with an EV membrane label, i.e., DiI, indicated the presence of miR-21/31 within EVs, whereas single MB fluorescence indicated ‘free’ miR-21/31 released from EVs. For Dicer detection [118], a hairpin-structured miRNA target for Dicer was labeled with a fluorophore–quencher pair at the two termini, thus having a quenched default state, and was loaded into EVs. Upon incubation of these EVs with cells, cleavage of the Dicer target caused the separation of the fluorophore–quencher pair, producing fluorescence, thereby enabling detection of Dicer activity on the miRNA target [118].

A modified calcium chloride transfection method including a heat shock step was recently developed by Zhang et al. [84] for miRNA post-loading of EVs. Calcium chloride transfection methods are well established for introducing exogenous DNA into mammalian cells [198] and heat shock was used to render the EV membranes more fluid, i.e., similar to the cellular plasma membrane fluidity to facilitate transient membrane destabilization and access for exogenous molecules to the EV lumen [199]. EVs loaded with miR-15a by this method were able to modulate the expression of target genes in recipient cells. Importantly, this method resulted in a similar loading efficiency as with the widely used electroporation method, but without the need for specialized electroporation equipment.

pH gradient modification of EVs (Fig. 3-7) was recently used to load miRNAs into EVs post-isolation [119]. Vesicles with an acidic internal compartment possess ion-trapping properties for weak base drugs. This means that weakly negatively charged small molecules can be effectively encapsulated in liposomes in the presence of a transmembrane pH gradient [200–203]. Based on this principle, by sequential dehydration with ethanol and rehydration in an acidic environment, followed by dialysis in a neutral buffer, a pH gradient was established between the EV lumen and its surrounding medium. By optimizing temperature, incubation time, and pH parameters, optimal miRNA-loading conditions were determined. EVs that were loaded with pro-inflammatory miR-146 by pH gradient modification were shown to dose-dependently induce macrophage inflammatory protein-2 production in

macrophages [119]. In the same study, this method was used to load EVs with siRNAs and ssDNA.

2.1.3. mRNA

In addition to the loading of EVs with small RNA molecules, including siRNA and miRNA, a growing number of papers shows that much larger, full-length mRNAs can also be loaded into EVs. However, due to preferential enrichment of short RNAs in EVs, loading of large RNAs such as full-length mRNA is still a challenge [124,204]. Wang et al. [120] loaded EVs with HChrR6 mRNA, which encodes a prodrug-activating enzyme, through the transient transfection of EV-producer cells with the mRNA. Co-administration of HChrR6 mRNA-EVs with the prodrug resulted in growth inhibition in cancer cells in an orthotopic breast cancer model in mice. Furthermore, EVs have been successfully loaded with Cre mRNA following stable transfection of EV-producer cells with Cre-encoding pDNA [121]. Alternatively, electroporation has been applied for loading mRNAs into EVs [123].

Although mRNAs can be successfully loaded into EVs, they are subject to rapid degradation in recipient cells [121,205]. Also, it was shown that in EVs derived from pDNA-transfected producer cells, the pDNA instead of the mRNA was the main contributor to the protein expression in recipient cells [106,136]. Moreover, co-loading of the recombinant protein that is being synthesized by the EV-producer cells upon their transfection with mRNA (or pDNA) may also occur. For example, Mizrak et al. [122] could load both mRNA and protein of cytosine deaminase (CD)-uracil phosphoribosyltransferase (CD-UPRT) into EVs by pDNA transfection of producer cells. Consequently, it becomes difficult to know the underlying reason for the change in protein expression in recipient cells. Overall, it should be noted that the phenotypic changes induced by EVs in recipient cells are mediated by the total arsenal of biomolecules present within the EVs, including proteins, DNA, and RNA, as well as lipids.

To stimulate the loading of RNAs, Hung and Leonard [124] fused the *Emesvirus zinderi* (MS2) bacteriophage coat protein to an EV-associated protein and decorated the cargo RNA with the MS2-binding RNA hairpin (Fig. 4-5 and -6). Fusion of the RNA-binding protein to vesicular stomatitis virus glycoprotein (VSVG), which is typically present in the plasma membrane, resulted in a 40 times higher RNA packaging level in EVs (specifically MVs). Loading was particularly efficient for short RNAs (<0.5 kb). Intriguingly, a 25 nt zipcode sequence was identified in mRNAs, which is a binding motif for miR-1289 and causes enrichment of mRNA into EVs [125] (Fig. 4-7 and -8). Although effective, this strategy demands very high cytosolic mRNA expression levels in order to achieve sufficient EV loading.

Recently, an innovative technique called cellular nanoporation was developed by Yang et al. [126] to increase mRNA loading into EVs. A monolayer of producer cells was cultured above a chip surface containing nanochannels (ideally 500 nm in width for cells 10–20 μm in size), enabling the passage of transient electrical pulses to generate pores in the cell membrane, which allowed extracellular pDNA to enter the cell cytosol. Concomitantly, the formation of pores caused an increase in intracellular calcium levels, which induced an increase in MVB formation and exosome production, with exosomes containing the pDNA-encoded mRNA. Using nanoporation, cells generated a 50-fold higher EV yield and a 10^3 times increase in EV mRNA content compared to bulk electroporation, with the majority of the mRNA present in exosomes rather than MVs [126]. This approach greatly simplifies the loading of mRNAs into EVs because it obviates the need to either incorporate mRNA-binding sequences in EVs through genetic engineering of producer cells or perform post-loading of isolated EVs with the mRNA (Fig. 4-9).

Although attractive, loading mRNA into EVs is yet an underdeveloped strategy for therapeutic advance. More knowledge about basic biology of EV biogenesis and mRNA sorting is required to improve mRNA loading into EVs. In this regard, it was recently shown that a neuronal protein called Arc which is evolutionarily related to viral transposon Gag proteins, can form virus-like capsids in EVs [206]. Additionally, Arc shows RNA-binding capacity toward its own transcript as well as cytosolic mRNAs, consequently, loading them into EVs. These EVs with Arc capsid structures and bound mRNA can transfer mRNA to other neurons where they can be actively translated [206]. Hence, Arc protein is a promising candidate for efficient mRNA loading and delivery. Tagging mRNAs with EV-tropic sequences could also present a useful strategy for EV loading, as exemplified recently by Yamashita et al. [207] They identified a typical RNA sequence in EVs by a SELEX method, i.e., several rounds of transfecting cells with a pool of 80 base RNAs, isolating EVs from transfected cells, and extracting RNAs from EVs. Such EV-tropic RNA sequence could be fused to mRNA cargo of interest to facilitate mRNA loading into EVs.

2.1.4. DNA

Most EV-based therapeutics involve RNA as the species of interest; however, some studies have shown that DNA can be loaded and transferred to acceptor cells via EVs. This is of interest because DNA offers a more stable and highly amplifiable alternative to RNA therapeutics and has potential for permanent correction of genetic disorders via genomic incorporation [208].

In a pioneering study, Kanada et al. [106] demonstrated pDNA loading into EVs (both exosomes and MVs) by transient transfection of producer cells with pDNA [106]. The resultant protein expression in cells that received those EVs turned out to be elicited by MV-mediated DNA transfer, while exosome-mediated DNA delivery led to rapid degradation of the DNA without eliciting a functional response. Moreover, pDNA was found to be more efficiently loaded into MVs compared to exosomes. Lamichhane et al. [128] used electroporation to load EVs with pDNA and observed that DNA encapsulation into EVs is generally inefficient, while being dependent on DNA size and conformation. Specifically, linear DNA (<1 kb) was more efficiently loaded into EVs than longer linear DNAs and non-linear pDNA. These findings suggest that MVs rather than exosomes are better suited for the delivery of DNA. However, DNA-loading capacity into EVs was generally very low, irrespective of the method of DNA incorporation (i.e., transient transfection of producer cells or electroporation of EVs).

Recently, the DNA-loading limit was greatly extended by using a specialized electroporation method on megakaryocyte MVs [129]. Remarkably, >3,000 copies of ~6 kb and 4,000 copies of 3.5 kb pDNA could be loaded into megakaryocyte MVs. This is the highest DNA-loading efficiency reported for MVs, exceeding by far the numbers suggested in other studies [106,128]. Thus, this particular electroporation strategy might be of advantage for loading DNA into EVs. Moreover, the loaded DNA was successfully delivered in recipient cells. Interestingly, co-incubation of cells with polybrene and MVs resulted in 84% MV-positive cells and high functional delivery of pDNA (determined from mRNA and protein levels), suggesting that polybrene might play a stimulatory role in EV uptake. Of note, considering the possibility that (negatively charged) pDNA is present at the surface of the EVs, the positively charged polybrene may serve to reduce the electrostatic repulsion between the DNA and the cell surface, thus promoting uptake [209]. Unfortunately, data on the surface charge of the MVs before and after electroporation with DNA were not provided. Using super-resolution structured illumination microscopy, intranuclear pDNA delivery was revealed together with the presence of the encoded protein (here, GFP) at the endoplasmic reticulum/Golgi region.

Minicircle DNA is a circular expression vector. Compared to the more conventional pDNA it is smaller, because it lacks the prokaryotic plasmid backbone, while being episomally stable [210]. Consequently, minicircle DNA induces higher and more prolonged protein expression compared to its plasmid counterpart [211]. DNA loading of MVs through transient transfection of EV producer cells was two-fold more effective with minicircle DNA compared to pDNA, while the resulting MVs elicited a 14 times higher functional outcome (luciferase expression) in acceptor cells [127]. Similarly, apoptosis in tumor cells was more efficiently induced *in vitro* with EVs carrying minicircle DNA encoding the thymidine kinase/nitroreductase fusion protein for dual prodrug therapy than with EVs carrying pDNA. When MVs carrying minicircle DNA or pDNA were injected in xenografts in mice, tumor size was greatly reduced only in mice treated with MVs carrying minicircle DNA. This suggests that the advantages of minicircle DNA over pDNA are even more pronounced *in vivo*.

Strategies other than to enhance DNA loading and stability could also be effectively employed to achieve better functional DNA delivery. For example, attaching a nuclear localization signal to the DNA can facilitate DNA localization to the nucleus, consequently increasing effective protein expression [212].

2.1.5. Protein

With intracellular delivery of DNA and RNA subsequent transcription and (inhibition of) translation is needed in order to effectively regulate the target protein expression level in cells. Moreover, stable integration of the DNA into the host genome may result in insertional mutagenesis [213]. Therefore, direct protein delivery is increasingly gaining popularity as a safe therapeutic method. Several reports have shown successful EV-mediated protein delivery and various methods have been developed to increase the protein payload in EVs and direct them to the therapeutic target.

Exogenous EV loading has been used to load proteins into EVs. Nakase et al. [130] showed that saporin, a small (30 kDa) ribosome-inactivating protein that induces cytotoxicity by inhibiting protein synthesis, was successfully loaded into EVs by electroporation. They successfully optimized the electroporation protocol to reduce protein aggregation. Saporin-loaded EVs were shown to induce considerable cytotoxicity in human pancreatic adenocarcinoma cells.

Other exogenous EV loading strategies include saponin treatment (Fig. 3-8), extrusion (Fig. 3-9), rapid freeze (−80°C)/thaw (RT) cycles (Fig. 3-10), and sonication [87]. Saponin is a mild detergent that permeabilizes cell membranes, enabling encapsulation of cargoes into EVs [214]. Extrusion works by repeatedly forcing a mixture of EVs and cargo through small pores, which causes collapse of the EV membranes and association with the cargo [215]. Freeze-thaw cycles cause cell membrane permeabilization due to the expansion of water upon freezing. It is commonly used to encapsulate drug molecules in lipid bilayer vesicles, i.e., liposomes [216,217].

Comparison among these methods for loading antioxidant catalase revealed that sonication, extrusion, and saponin treatment display high loading efficiency, owing to the extensive rearrangement of the lipid bilayer rendering it more permeable to cargo loading. Moreover, saponin-treated EVs showed highest *in vivo* catalase activity. Nonetheless, careful consideration must be given to choosing the loading method, as most *ex vivo* techniques lead to substantial deformation of the EV membrane, potentially disrupting EV integrity, leading to a loss of immune-privilege, and reduced effectivity.

Most recently, toward minimizing EV membrane disturbance, single-stranded DNA conjugated to cholesterol was used to load bioactive proteins on EVs (Fig. 3-11) [131]. Such a DNA-cholesterol tether can be easily inserted into the EV lipid bilayer and when modified with biotin allows for the conjugation of streptavidin-modified cargoes. FasL, an immunomodulatory protein that plays an important role in programed cell death and immune homeostasis [132], was modified with streptavidin [133] and attached to EVs containing biotin-modified DNA cholesterol tethers via streptavidin–biotin interaction. These FasL-EVs effectively induced dose-dependent apoptosis in T cells. Although highly effective, further research is required to evaluate the possible effects of surface conjugation of cargo on the intrinsic homing ability and cell-specific targeting ability of the EVs.

Proteins can be endogenously loaded into EVs through the overexpression of the protein in EV producer cells by transfection or transduction with protein-encoding pDNA. Aspe et al. [134] showed that Survivin-T34A mutant protein involved in abrogating therapeutic resistance in pancreatic cancer was loaded into EVs by its overexpression in producer cells. EVs containing the mutant protein successfully enhanced pancreatic cancer cell death *in vitro*. Similarly, heat shock protein 70 (HSP70) was efficiently packaged in EVs. Pathogenic protein aggregation was significantly decreased upon administration of HSP70-loaded EVs to cellular and drosophila models of Huntington's disease [135]. Similarly, overexpression of the prodrug-activating CD-UPRT enzyme fusion in cells led to its loading into EVs. Intratumoral delivery of CD-UPRT EVs in human schwannoma tumors present in sciatic nerve of nude mice combined with prodrug 5-fluorocytosine treatment resulted in significant tumor regression [122].

As a result of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, chloride channel activity is impaired in cystic fibrosis (CF) patients, which leads to thick mucus secretion from epithelial cells, causing a.o. reduction in lung capacity and higher susceptibility toward infections [218–221]. EVs collected from CFTR-overexpressing cells were able to correct the membrane chloride channel activity in CFTR-mutant cells [136]. Importantly, this activity was maintained for 3 days and could be detected even at day 5, while it was shown to be the result of newly synthesized proteins from CFTR mRNA that was present in the EVs. This, for the first time, demonstrated the potential of EV-mediated therapy for treatment of a genetic disorder. Interestingly, MVs consistently contained 10- to 20-fold higher amount of CFTR cargo (protein and mRNA) than exosomes and were able to elicit a higher therapeutic response. Moreover, MVs presented multiple CFTR protein bands on Western blot, suggesting that MVs, in addition to the intact cargo protein, may also export protein degradation products. Finally, the EVs were shown to contain viral CFTR-encoding DNA, which was used to transduce the producer cells. Although viable vector was not obtained from the EVs, the DNA could still be transcribed in the recipient cells and led to expression of the CFTR protein.

In contrast to the passive loading of overexpressed proteins into EVs, EVs can be actively loaded with proteins a.o. via the expression of cargo protein fusions with EV-associated proteins. Cellular overexpression of ovalbumin (OVA) antigen fused to CD63 resulted in the secretion of OVA-loaded EVs (Fig. 4-10) [137]. OVA-specific $CD4^+$ and $CD8^+$ T-cells were induced in naïve mice following immunization with purified OVA-EVs.

Alternatively, active loading of EVs can occur through protein–protein interaction. Proteins containing late (L) domains are involved in the recruitment of proteins to MVBs and plasma membrane, for sorting into exosomes and MVs, respectively. L domains in proteins contain specific motifs that mediate protein–protein interactions [222–224] (Fig. 4-11). Sterzenbach et al. [138] tagged a Cre recombinase with a WW tag that specifically interacts with the L domain on Ndfip1 through three L-

domain motifs (PPxY) and as a result gets sorted into intraluminal vesicle [225]. Using this strategy, authors showed that Cre recombinase could be efficiently loaded into exosomes and transferred into recipient cells. Such EV-mediated delivery of Cre resulted in Cre-specific recombination in mouse embryonic fibroblasts *in vitro* as well as multiple regions of the mouse brain *in vivo*.

Arrestin domain containing protein 1 (ARRDC1)-mediated MVs (ARMMs) constitute a type of MVs formed by ARRDC1- and TSG101-mediated budding of the plasma membrane [226–228]. ARRDC1, like Ndfip1 protein, interacts with WW domains of proteins belonging to the neuronal precursor cell-expressed developmentally downregulated 4 family (Fig. 4-12) [138]. It was recently shown that NOTCH receptors were actively recruited into ARMMs and could bring about NOTCH signaling in cells that received ARMMs. A protein of interest fused to ARRDC1 is actively packaged into ARMMs and can be delivered into target cells (Fig. 4-13). This strategy was successfully used to deliver chimeric ARRDC1-p53 protein to tumor cells, which resulted in tumor suppression *in vivo* [139]. Furthermore, using the interaction between the chimeric protein of ARRDC1 fused to Tat1 peptide (which specifically binds to the stem-loop-containing *trans*-activating response (TAR) element) and p53 mRNA fused to TAR, researchers loaded the p53 mRNA into ARMMs. These ARMMs successfully delivered functional mRNA in recipient cells [139] More complex molecules such as protein/RNA complexes were also loaded using this strategy [139]. Expression of a WW-Cas9/sgRNA encoding construct in donor cells led to incorporation of WW-Cas9/sgRNA complexes into ARMMs and efficient delivery to recipient cells. Using sgRNA directed against GFP, GFP downregulation indicated successful gene editing in recipient cells.

An innovative protein–protein interaction exploiting a light-inducible system derived from *Arabidopsis thaliana* called EXPLORs (exosomes for protein loading via optically reversible protein–protein interactions) was recently used for protein loading into EVs [140]. With this method, a blue light–dependent reversible protein–protein interaction [229–231] between photoreceptor cytochrome 2 (CRY2) (fused to a cargo protein) and CRY-interacting basic-helix-loop-helix 1 (CIB1) (fused to the EV-associated protein CD9) was established in producer cells. Blue light irradiation–induced interaction of CRY2 and CIB1 consequently led to the incorporation of cargo into EVs (Fig. 4-14). Administration of these vesicles resulted in efficient cytosolic delivery of the cargo protein and its function *in vitro* and *in vivo*. Importantly, in the absence of blue light the cargo proteins in EXPLORs are no longer bound to CD9 and thus 'free' in the EV lumen, which aided effective cargo release from EVs. When compared to EVs that were generated with the commercial protein-loading system XPack (System Biosciences), EXPLORs showed approximately six-fold higher cytosolic delivery of the cargo protein. However, this technology warrants further improvement as the exposure of a strong blue light resulted in the aggregation of CRY2 proteins which could disturb the desired protein–protein interaction and consequent cargo loading, limiting the loading efficiency.

2.1.6. Lipids

EVs contain various lipids in their membrane and lumen, such as cholesterol, sphingomyelin, lysophosphatidylcholine (LPC), arachidonic acid and other fatty acids, prostaglandins, and leukotrienes [40], which have structural as well as informational functions. For example, prostaglandins [232] and leukotrienes [233] are bioactive lipids known for their role in cell–cell signaling. LPC plays a role in lymphocyte chemotaxis and dendritic cell differentiation. Consequently, (LPC-containing) EVs have the potential to boost immune response [234]. In fact, various lipids contained in EVs have shown immunomodulatory functions and could be interesting candidates for the use of EVs for immunomodulation *in vitro* and *in vivo* [234].

Exogenous loading of EVs with lipids is relatively easy, because of the spontaneous incorporation of lipids in the EV membrane, enabling EV-mediated lipid delivery to target cells. For example, EVs isolated from HER2 expressing cells were loaded with phospholipids through fusion with liposomes as induced by repeated freeze-thaw cycles. Cellular uptake of the resultant 'hybrid' EVs was two-fold more efficient compared to control EVs [141]. For imaging purposes, EVs were labeled with fluorescent lipophilic dyes. However, this has been shown to increase EV size which may disturb EV function [235]. In addition, EVs have been loaded with cationic lipids to form hybrid exosomes [142], primarily to promote the release of EV cargo into the cell cytosol. In general, although easily amenable to lipid loading, the use of EVs as therapeutic lipid transporters still remains limited. Knowledge on the mechanisms of natural lipid loading in EVs and the function of the distinct lipid signatures of different EV subtypes may help to develop non-disturbing exogenous lipid-loading techniques.

2.1.7. Small molecules

Poor pharmacokinetic, low efficacy, and toxic side-effects cause poor translation of small molecule drugs to a clinical setting [236,237]. EVs provide an opportunity for small molecule encapsulation and solubilization, protection against degradation, prolonged circulation, improved tissue specificity and tissue retention, thus making attractive carriers for small molecule drugs [238]. Various chemotherapeutic drugs and naturally bioactive compounds have been successfully loaded into/onto EVs by similar methods as for biomolecules.

The simplest strategy is to load small molecule drugs, including anti-inflammatory, chemotherapeutic, and antioxidant agents, into isolated EVs by simple co-incubation. For example, curcumin, a polyphenolic hydrophobic compound with antioxidant, anticancerous, anticoagulant, and anti-inflammatory properties, was efficiently associated with EVs and reduced inflammation in various tissues irrespective of the route of administration [143–145]. Doxorubicin, a widely used chemotherapeutic agent, was loaded in macrophage- and blood cell-derived EVs by passive incubation and displayed antiglioma activity [146]. Similarly, the antitumor agent paclitaxel [147] when loaded into bovine milk–derived EVs showed antitumor activities [148]. Importantly, the oral availability of paclitaxel [149] was greatly enhanced by its EV incorporation along with maintaining stability under harsh gastrointestinal fluid conditions [148]. Furthermore, paclitaxel loaded in embryonic stem cell–derived EVs showed improved curative effects in glioblastoma treatment [150]. Of note, the loading efficiency of small molecule drugs into EVs by passive incubation depends upon their degree of hydrophobicity. A range of chemopreventive agents with varying lipophilicity showed 10–40% loading differences in milk-derived EVs, and high release efficiency in recipient cells [151]. Sonodynamic therapy (SDT), i.e., focused ultrasound-mediated site-specific drug activation, is a recent advance in cancer therapy. Chemo-sensitizer drugs that are used in SDT have also been loaded into EVs. For example, sinoporphyrin sodium, a porphyrin sensitizer for SDT theranostics [152] when associated with EVs displayed efficient release in tumor cells upon ultrasonic activation and 10-fold higher metastatic inhibition than in free form [153]. Moreover, encapsulation in tumor-derived EVs resulted in homotypic delivery. To enhance the loading of the highly hydrophilic neurotransmitter dopamine into blood-derived EVs, high-concentration dopamine solutions were first generated by means of complexation with vitamin C. The resulting dopamine-EVs showed higher therapeutic efficacy and lower systemic toxicity than free dopamine in a Parkinson's disease mouse model [154].

In addition, to enhance the exogenous loading of EVs with small molecules, electroporation has been employed. Electroporation of EVs in a solution with doxorubicin resulted in a 20% encapsulation efficiency, while mice treated with dox-EVs showed reduced tumor growth and cardiotoxicity compared to mice receiving the free drug [155,156]. Enkephalin, a neurotransmitter involved in pain reduction, analgesia, and euphoria [239] loaded into EVs via electroporation, resulted in neuroprotection and neural recovery in ischemia–reperfusion injury [157]. Curcumin was similarly loaded into EVs for use in glioma treatment [158]. As an alternative approach, sonication has been employed for loading the anticancer agents triptolide [162] and paclitaxel [159,160].

Cells which were incubated with the chemotherapeutic drug methotrexate have been shown to expel the drug from the cells through the encapsulation in EVs. Importantly, these EVs showed higher cytotoxicity in acceptor cells compared to the free drug, without the typical side-effects of the free drug [163]. Similarly, pharmaceutically relevant levels of the chemotherapeutic drugs paclitaxel [164–166], imatinib [183], doxorubicin [163,167], curcumin [168], and 3,3′-diindolylmethane [169] have been loaded into EVs by incubation of cells with the drugs.

Recently, an innovative approach was taken in which EVs were isolated from cells that were incubated with fusogenic liposomes containing hydrophobic and hydrophilic small molecules. EVs thus obtained contained the hydrophobic small molecules (DiI) and to a certain extent hydrophilic molecules (calcein) and showed potential to penetrate *in vitro* tumor spheroids and *in vivo* tumors [170]. This work demonstrated the intriguing possibility of combining advantageous properties of natural nanoparticles (EVs) with synthetic ones (liposomes) for drug delivery purposes. Of note, this procedure was further extended to introduce azide-lipids into EV membranes, which could be further equipped by click-reaction (see Section 3.1) with functional entities, including drugs, fluorophores, and ligands, in the latter case generating EVs loaded with small molecules and decorated with targeting moieties [171].

In general, the choice for a specific loading method is mainly governed by the nature of the drug, i.e., hydrophobicity and charge and the extent to which the method imposes damages on EVs [161]. Different loading methods can greatly differ in outcome in terms of encapsulation efficiency, effect on EV composition, and processing time. In a comparative study of methods employed for loading EVs with paclitaxel, Kim et al. [159] showed that, among incubation, extrusion, sonication, electroporation, and freeze and thaw methods, sonication showed maximum loading capacity (29%), while electroporation reached 5.3% loading and incubation achieved minimum loading of 1.4%. The high loading capacity with sonication was a result of considerable reorganization of the EV membrane, involving changes in microviscosity, rendering it more permissive to drug loading. Importantly, EV integrity was restored within 1-h incubation at 37°C and its protein and lipid composition was not affected. In another comparative study [161], Fuhrmann et al. investigated the effect of porphyrin hydrophobicity on loading efficiency via passive (incubation) and active (electroporation, extrusion, saponin-assisted, hypotonic dialysis) loading methods. The most hydrophobic porphyrin was efficiently loaded into EVs via passive loading, while electroporation was shown to substantially enhance the loading of the more hydrophilic porphyrins. Interestingly, the lipid composition of the EVs seemed to influence loading efficiency, because EVs isolated from different cell types showed different loading efficiency under the same loading conditions. These differences could not be related to differences in cholesterol content of the different EVs, which was a first guess based on the assumption that cholesterol increases bilayer rigidity. However, because membrane fluidity is dependent on temperature, and EV loading was performed at RT (except for extrusion), it would be of interest to investigate the effect of temperature on loading efficiency. In an attempt to increase the loading of a more hydrophilic porphyrin, saponin co-incubation and hypotonic dialysis (Fig. 3-12) were shown to increase the loading efficiency by 11-fold compared to passive loading. However, EVs loaded via hypotonic dialysis showed poor cellular uptake, preventing porphyrin-mediated phototoxicity following laser irradiation.

Taken together, EVs prove to be advantageous for carrying small molecule drugs. They enhance the solubility of hydrophobic molecules, thus increasing their bioavailability and decreasing the dose required for achieving a therapeutic effect. Furthermore, EVs can promote the cellular uptake of hydrophilic drugs. However, the drug loading method should be carefully chosen, taking into account the degree of hydrophobicity of the drug and the EV cell source in order to achieve maximum loading efficiency. Moreover, in addition to loading efficiency, cell uptake and intracellular drug release need to be investigated in order to decide on the best protocol for generating drug-loaded EVs.

3. EVs as drug delivery systems: unloading cargoes

Unloading the encapsulated cargo with cellular and subcellular precision is of utmost importance for an EV therapeutic to be successful. Most EV formulations despite their proven *in vitro* uptake by target cells, accumulate primarily in liver or spleen under *in vivo* settings [155,240–242]. In an attempt to increase target cell specificity, various methods have been employed for improved targeted delivery to a desired cell type as well as enhanced cargo release, *in vitro* and *in vivo*. These methods are discussed below and illustrated in Fig. 5.

3.1. Enhancing EV uptake in target cells

In one of the early demonstrations, Alvarez-Erviti et al. [80] engineered brain-targeting EVs by fusing rabies virus glycoprotein (RVG) peptide to an EV-rich protein, i.e., lysosome-associated membrane protein b (Lamp2b). RVG peptide has high affinity toward acetyl choline receptors [243,244]. EVs isolated from RVG-Lamp2b expressing parental cells displayed RVG peptide on the surface and accumulated in the brain when injected intravenously. These EVs were successfully exploited for functional delivery of siRNA to the brain. This study paved the way for delivery of various biomolecules to the brain using RVG, while other peptide-Lamp2b fusions were used to target EVs toward other desired cell types. For example, a recent study [80] used RVG-EVs for delivery of miR-124 to the brain in a mouse stroke model. Similarly, mesenchymal stem cell–derived EVs modified with RVG led to reduced amyloid plaque deposition and neuroinflammation, and importantly, improved cognitive deficits in Alzheimer's disease model upon intravenous administration [245]. An alpha v integrin-specific iRGD peptide with tumor targeting properties, previously shown in prostate, breast, cervical, and pancreatic cancer models, was fused to Lamp2b. iRGD-decorated EVs showed enhanced tumor targeting efficiency in an *in vivo* breast cancer model [155]. Similarly, EVs displaying an ischemic myocardium targeting peptide [246] and a cardiomyocyte-specific peptide [247] fused with Lamp2b showed tissue-specific uptake. Altogether, these studies showed the general applicability of peptide-Lamp2b fusions for targeting purposes. Furthermore, larger proteins were also displayed at the EV surface using this strategy. A fragment of interleukin 3 displayed on EVs led to their increased uptake in chronic myeloid leukemia cells [183]. And the strategy has been extended to other EV-rich proteins. For example, the transmembrane domain of platelet-derived growth factor receptor was used as a display system for the GE11 peptide (YHWYGYTPQNVI) to target EVs to EGFR-overexpressing cancer

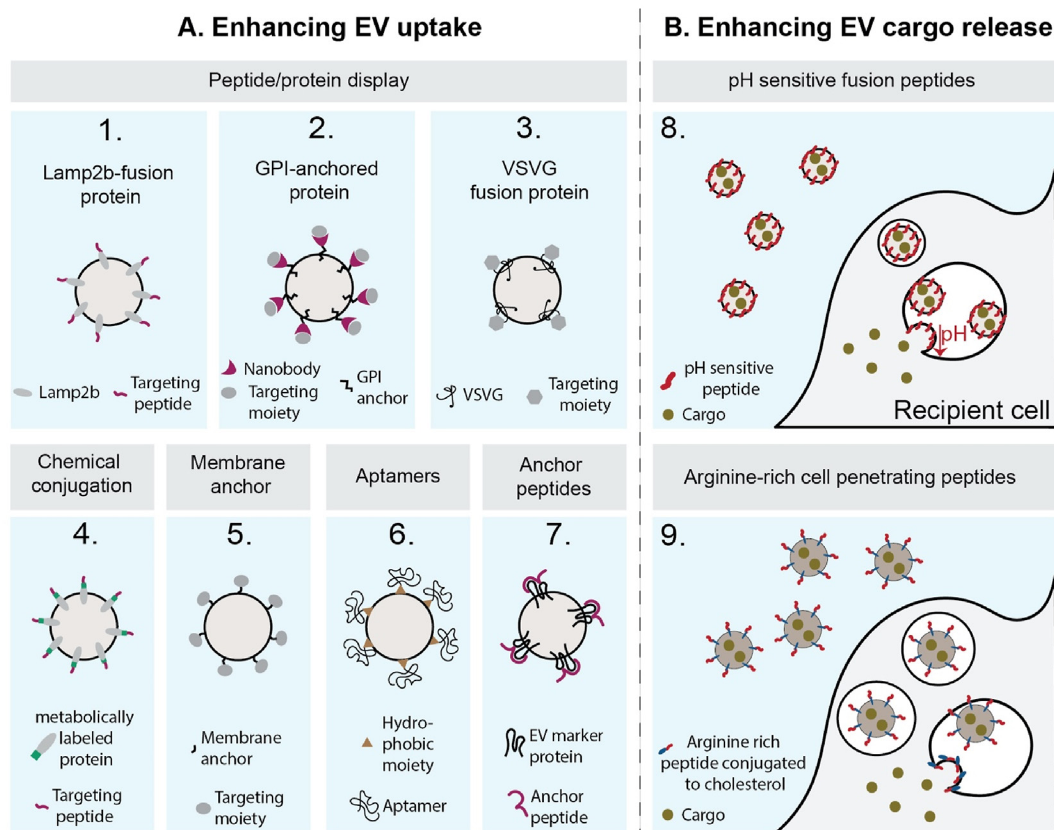

Fig. 5. Strategies to enhance cellular uptake of EVs and cargo release. (A) Enhancing EV uptake. 1. An EV-enriched protein, e.g., Lamp2b, is fused to a peptide that targets a specific cellular receptor. 2. A phosphoinositol (GPI) anchor signal peptide is fused to a nanobody against a receptor ligand. The ligand binds to the nanobody and targets a specific cellular receptor. 3. VSVG is fused to a protein that targets a specific cellular receptor. (Expression of VSVG only results in a broad cell tropism.) 4. A Clickable moiety (e.g., azide) is inserted into an EV membrane protein by metabolic labeling, followed by chemical conjugation of a clickable (e.g., alkyne-functionalized) targeting peptide post-isolation. 5. A hydrophobic membrane anchor conjugated to a targeting peptide is inserted into the EV membrane. 6. A hydrophobic moiety conjugated to an aptamer is inserted into the EV membrane. 7. An anchor peptide, which recognizes an epitope of an EV surface protein (e.g., the extracellular loop of CD63), conjugated to a targeting peptide is bound to EVs. (B) Enhancing EV cargo release. 8. A pH-sensitive fusion peptide is expressed in the EV membrane. Following cellular uptake of EVs through endocytosis and subsequent endosomal acidification, the fusion peptide mediates EV fusion with the endosomal membrane, facilitating cargo release. 9. Arginine-rich peptides promote the cellular uptake of EVs, thereby enhancing the chance of cargo release.

cells [101]. Alternative targeting moieties such as nanobodies have also been used for EV targeting. For example, taking an innovative approach by using the cell's own glycosylphosphatidylinositol (GPI)-mediated protein anchoring, Kooijmans et al. [248] showed that expression of a GPI anchor signal peptide fused to anti-EGFR nanobody resulted in nanobody-displaying EVs, which showed increased binding to EGFR-expressing cells under both static and flow conditions.

Recently, Meyer et al. [249] described the genetic manipulation of donor cells to express the VSVG, used in viral pseudotyping, which was incorporated into EVs. VSVG-pseudotyped EVs showed enhanced uptake by cells and broad cell tropism compared to EVs displaying VSVG without its ectodomain. By exchanging the VSVG ectodomain with specific disease-targeting molecules one could target specific tissues not only for therapeutic but also for diagnostic purposes [249].

The surface display of peptides and nanobodies on EVs shows promise in enhancing EV targeting to specific tissue types and, in turn, improving their therapeutic effect. Because the targeting efficiency of nanoparticles, including EVs, is dependent on the interaction between the targeting ligands and their cellular receptors [250], the receptor expression level and the route of entry in the desired recipient cells likely decide whether a particular targeting strategy is translated into the desired therapeutic effect [251]. Moreover, nanoparticles, although taken up efficiently in recipient cells may not exhibit therapeutic effects due to a lack of endosomal escape and consequent cargo delivery [252,253]. Besides, surface-displayed peptides may undergo proteolytic degradation during EV biogenesis (in endosomes) as well as after isolation and/or when administered *in vivo*. The glycosylation of peptides by adding the amino acid sequence GNSTM, an *N*-linked glycosylation sequon [254], has been shown to protect the targeting peptides from premature degradation during EV biogenesis and to result in enhanced display and function [255]. Still, genetic engineering strategies are often time-consuming and may lead to batch-to-batch variation [256,257], while genetic engineering of EV producer cells is required anew for each individual EV (surface) modification. To this end, functionalization of EVs post-isolation, via click chemistry or post-insertion of membrane anchors present potent alternatives.

Click chemistry, specifically copper-catalyzed azide alkyne cycloaddition (CuAAC), is a widely employed method for surface modification of biomacromolecules, including DNA, peptides, and antibodies [258–262]. The method comprises (relatively) mild reaction conditions, use of easily available reagents, and presents high efficiency of cross-linking [263,264]. In a two-step reaction, first, an alkyl group is chemically added to biomolecules, followed by their copper-assisted conjugation to azide-functionalized

moieties, resulting in biomolecules functionalized with the moieties. Using click chemistry, Jia et al. [158] attached neuropilin-1 targeting peptide (RGERPPR) to isolated EVs to produce glioma-targeted EVs. Following intravenous administration these EVs inhibited tumor growth, delayed tumor recurrence, and extended the survival of tumor-bearing mice. Cyclo (Arg-Gly-Asp-D-Tyr-Lys) peptide is used to target cells overexpressing $a_vß_3$ integrins, e.g., actively proliferating endothelial cells in glioblastoma multiforme (GBM), prostate cancer, and lung cancer. Due to its propensity toward endothelium, this peptide has also been used to cross the blood-brain barrier (BBB) [158,265]. However, it has been reported that over-modification (alkylation) of antibodies lowers their binding affinities, while minimal modification maintains antibody's biological functions [266]. Therefore, the number of alkyne modifications on proteins should be kept at a minimum to maintain their function following their modification through click chemistry. Smyth et al. [266] reported that 1.5 alkyne modifications for every 150 kDa of exosomal protein was compatible with EV function. Alternatively, exosomes were functionalized with azide prior to their isolation through the metabolic labeling of proteins, including EV proteins, in producer cells. To this end, cells were incubated with an azide-bearing amino acid analog of methionine or azide-containing saccharides to label newly synthesized proteins or glycans and glycoproteins, respectively. Subsequently, copper-free strain-promoted azide-alkyne cycloaddition (SPAAC) was used to functionalize isolated EVs. Using this strategy, Wang et al. [267] functionalized EVs with biotin to which a wide variety of other functional moieties could be easily added via streptavidin or avidin interactions. Taken together, click reaction-mediated EV functionalization is convenient, time and cost-effective, applicable to functionalize EVs post-isolation, and applicable for the conjugation of various kinds of (macro)molecules as well as mixtures of (macro) molecules, thus generating multifunctional EVs.

Due to the requirement of chemical reactions in CuAAC and SPAAC and the associated possibility of EV surface structure alterations [266], an alternative post-isolation functionalization approach encompassing the post-insertion of membrane anchors conjugated to targeting moieties, has recently been developed. For example, a lipidomimetic (octadodecyl) chain conjugated to hyaluronic acid was inserted into the EV membrane to achieve tumor targeting [268]. In another study, the lipid derivative DSPE-PEG was conjugated to anisamide to target the sigma receptor, which is characteristically overexpressed in lung cancer tissues [269]. Similarly, Zhu et al. [150] conjugated the RGD peptide to EVs using DSPE anchors. RGD peptide specifically targets α5β3 integrin, which is upregulated during angiogenesis, therefore abundantly present on actively proliferating endothelium in, e.g., glioblastoma tissue [158,270]. The RGD-EVs accumulated at the site of glioblastoma more efficiently than non-targeted EVs, demonstrating enhanced targeting ability due to peptide display. Alternatively, DSPE-PEG-RGD was incorporated into the EV membrane following incubation of parental cells in medium supplemented with the lipid-peptide conjugate, which resulted in its insertion in the plasma membrane and consequent loading in EVs [271]. Subsequently, these EVs were loaded with an azide derivative of a monosaccharide. The resulting EVs showed high endothelial interaction and were successfully used to metabolically label newly formed blood vessels, which were visualized following copper-free click chemistry with dibenzocyclooctyne fluorescein *in vitro* and *in vivo* in zebrafish. In another study using membrane insertion, DMPE-PEG-streptavidin was inserted into the EV membrane. This way EVs could be decorated with any biotinylated molecule, including fluorescent molecules and targeting antibodies [272].

Although antibodies and peptides remain to be the most commonly used targeting ligands on EVs, their surface display often requires complicated genetic engineering [83] and/or toxic chemical cross-linking reactions [273]. Moreover, their clinical applications are limited due to suboptimal specificity, batch-to-batch variation, immunogenicity, and low stability [274,275]. A recent advance in EV surface functionalization was made through the use of aptamers, also known as chemical antibodies. Aptamers are short, single-stranded DNA or RNA molecules that possess specific 3D structures and therefore have the ability to specifically interact with a target, including proteins, peptides, and carbohydrates [276,277]. Owing to their non-immunogenicity, high specificity, thermal stability, low toxicity, and easy synthesis, they offer great promise as durable and specific targeting ligands [278,279].

Aptamers are generally conjugated to a hydrophobic moiety such as cholesterol for facile incorporation and display at the EV membrane [280]. For example, using a cancer-specific aptamer conjugated to a diacyl lipid tail, Zou et al. [281] demonstrated that Dox-loaded EVs decorated with an antiprotein tyrosine kinase 7 (PTK7) aptamer (sgc8) showed enhanced uptake and toxicity in PTK7-expressing cancer cells. Importantly, EV functionalization was achieved by simple mixing of the lipid-aptamer conjugate with the EVs for half an hour at 37°C, showcasing the ease of use of this approach. Pi et al. used cholesterol to insert an arrow-shaped RNA aptamer into EVs: cholesterol attached to the tail of the RNA arrow led to RNA surface display, whereas its attachment to the arrowhead led to RNA loading inside EVs. This way, the RNA aptamer was used to function as a targeting ligand and to enhance the loading of EVs with siRNA [282]. The EVs successfully delivered therapeutic small RNAs in targeted cancer cell types and inhibited tumor growth *in vivo*. More recently, Shamili et al. [283] used an aptamer with affinity for myelin to functionalize EVs. The aptamer-EV bioconjugate promoted the proliferation of oligodendroglial cells *in vitro* and suppressed inflammation as well as demyelination *in vivo*. Yerneni et al. [131] used an aptamer AS1411 against nucleolin, a protein highly expressed on cancer cells, to achieve specific targeting toward cancer cells. Altogether, the studies highlight the potential of aptamers to redirect EVs to target cell types. With concerted efforts from various disciplines, including biology, chemistry, and medicine, aptamer-conjugated EVs pose a promising possibility for the development of the next-generation delivery vehicles that are highly specific, stable, easily synthesized, and customizable.

Recently, EV functionalization was greatly simplified by identifying anchor peptides that could decorate EVs with cargo and targeting moieties without the need for genetic modification, harsh chemical reactions, or lipid conjugation. Using phage display against the large extracellular loop of CD63, an EV marker protein, Gao et al. identified CP05 peptide (CRHSQMTVTSRL). EVs decorated with CP05 conjugated to exon-skipping antisense oligonucleotides (ASOs) for the treatment of Duchenne muscular dystrophy, showed, in comparison to CP05-ASOs, an 18-fold increase in dystrophin expression in the quadriceps of mice in a Duchenne muscular dystrophy model [284]. Additional decoration with CP05 conjugated to a muscle-targeting peptide further increased dystrophin expression. Similarly, EVs with surface-displayed brain-targeting peptide RVG or hepatocellular carcinoma-binding peptide SP94 efficiently distributed to brain and liver, respectively [284]. CP05 can be used to functionalize any CD63-positive EV with targeting moieties irrespective of its origin, highlighting the broad applicability of the approach. This way, patient-derived EVs can be easily modified for therapeutic purposes, avoiding immunological reactions owing to their autologous nature.

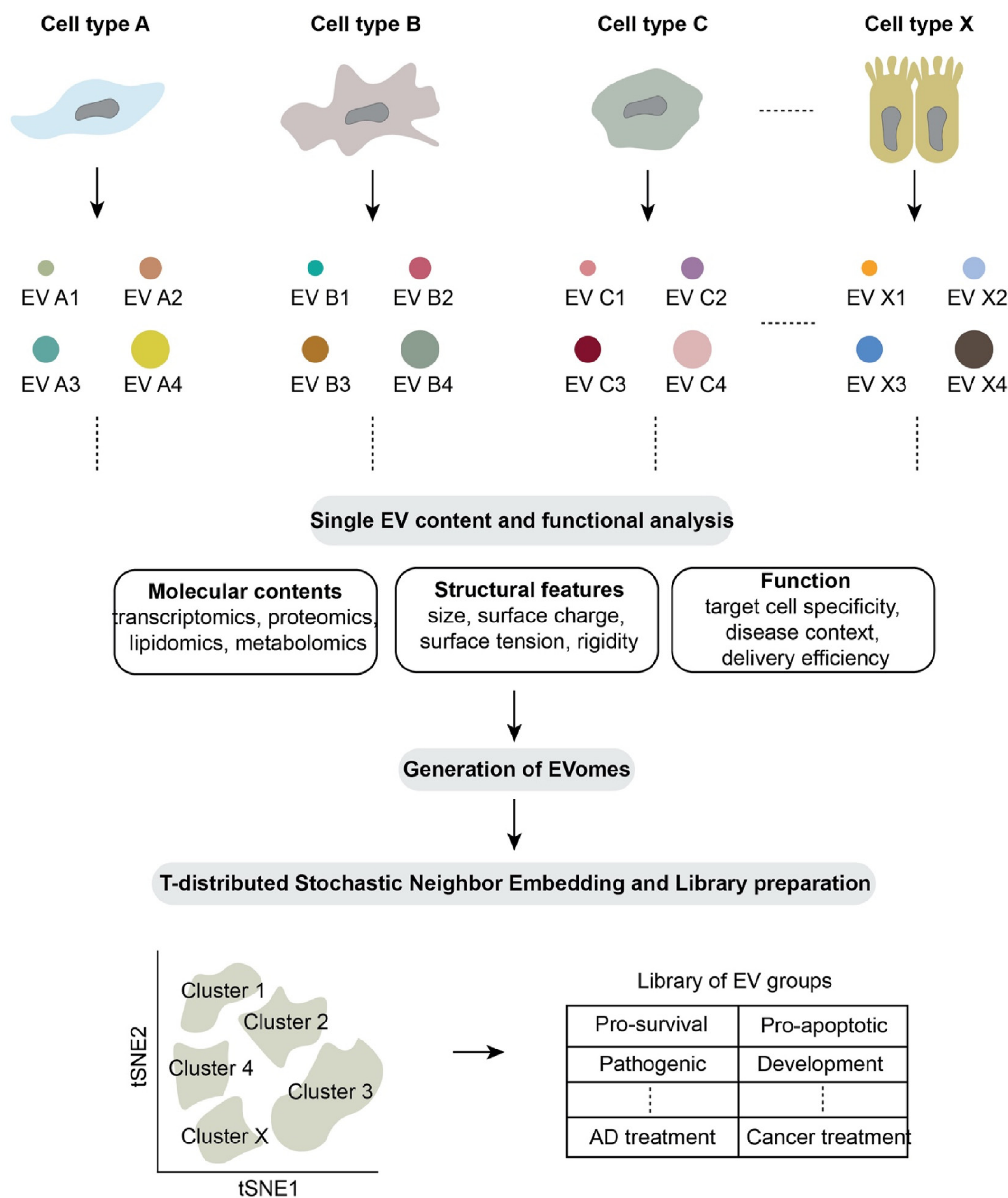

Fig. 6. Toward EV-omics and application-specific EV libraries. EVs from a variety of physiologically relevant donor cells are isolated, followed by a comprehensive molecular, functional, and structural characterization of the EVs and their content by employing state-of-the art 'omics' technologies, biophysical methods, and functional assays. This collection of EV features could be used to build a mathematical model such as tSNE, which could be used to cluster features that are connected to a specific function, as well as to extract classifying features between clusters. The resultant EV subtyping can be tested experimentally by manipulating EV identity and determining its effect on EV function. At a later stage, the tSNE model could be used to generate an application-specific EV library set.

3.2. Enhancing EV cargo delivery in target cells

Achieving therapeutic activity depends not only on efficient EV delivery to target cells and high cargo content, but also on adequate EV cargo release in recipient cells. Most studies have taken an indirect approach to evaluate functional cargo delivery by EVs by probing phenotypic modulation in recipient cells. Recently, Joshi et al. [285] showed that approximately 25% of internalized EVs in HEK293T cells undergo pH-dependent fusion with endosomal membranes, i.e., back-fusion, to release their cargo. This suggests that functional delivery of EV content may be further enhanced to improve therapeutic outcome. To this end, various approaches have been utilized. Morishita et al. took a pre-isolation approach by engineering murine tumor cells to produce EVs carrying a pH-sensitive fusion protein (GALA) on their membrane. Following GALA-EV endocytosis and endosomal acidification, GALA fused with the endosomal membrane resulting in endosomal leakage. Consequently, the EV cargo was released into the cytosol of the recipient cell. Because many therapeutic molecules, including siRNA and mRNA, act in the cytosol, promoting the intracellular cytosolic delivery of these molecules by engineered EVs represents a promising delivery approach. Further studies are needed to analyze GALA-EV stability and performance when encapsulating therapeutic molecules. The compatibility of GALA peptide modification of EVs with the loading and release of therapeutic cargoes remains to be investigated [286].

In a post-isolation approach, arginine-rich cell penetrating peptides (CPPs) were displayed on EVs using a stearyl membrane anchor. The CPPs promoted the cellular uptake of EVs via macropinocytosis and enhanced the cytosolic delivery of a ribosome inactivating protein [248,287].

3.3. In vivo EV release

Despite rapid progress, effectively creating designer EVs with sufficient yield and brief pre-processing still remains challenging. To overcome these challenges, Kojima et al. [288] created EXOsomal Transfer into Cells (EXOtic) devices. A multipronged approach was used to achieve high EV yield, specific RNA packaging, target cell specificity, and efficient cytosolic delivery. *In vivo* implanted EV-producer cells were engineered to overexpress a STEAP3-SDC4-NadB production booster, which led to an approximately 15-fold increase in EV yield. Additionally, an mRNA packaging plasmid (encoding L7Ae fused to CD63), an mRNA of interest carrying a C/D_{box} in the 3′ untranslated region which interacts with L7Ae, a cytosolic delivery facilitator gene (encoding constitutively active connexin 43), and a targeting plasmid (encoding RVG-Lamp2b) were introduced into producer cells to generate EXOtic devices. Implanted designer cells were used to release EVs for catalase mRNA delivery to brain cells to reduce neurotoxicity and inflammation in *in vitro* and *in vivo* Parkinson's models.

Another innovative approach for EV loading is the *in vivo* production of cargo-loaded EVs through DNA inoculation. Recently, Bonito et al. [289] developed an EV-based immunization therapy capable to elicit tailored immune responses. This strategy is based on an *in vivo* intramuscular DNA inoculation to produce host-derived engineered EVs. Intramuscular injection of a DNA vector encoding modified Nef (lentiviral protein) fused with HPV E7 (human papillomavirus oncoprotein) into a host animal induced the release of muscle cell-derived EVs carrying Nef^{mut}E7 which induced an HPV E7-specific immune response. Of note, muscle cells are the only cell type that can be *in vivo* transfected with naked DNA without the need for a vector, which makes them ideal for transient *in vivo* production of loaded EVs. However, it may prove difficult to control the level of EV production, which may in the case of immunization trigger a too strong immune response, converting therapeutic EVs into harmful entities. Therefore, further standardization of this technique is warranted to allow for its safe clinical application.

4. Conclusions: the holy grail of pharmaceuticals

EVs largely conform to the holy grail of pharmaceuticals and drug delivery systems, given that they are easily produced, modifiable, and show organotropic behavior. The clinical success of pharmaceuticals depends on a multitude of features including the following: (1) easy synthesis and modification, (2) large-scale production, (3) easy and long-term storage, (4) convenient route of administration to the patient, (5) long-term stability in circulation, (7) organ specificity, (8) cellular and subcellular targeting with access to the right cellular machinery for proper functioning, (9) cost-effectiveness, and (10) safety. Although the comprehensive advancement in EV research in recent decades has shown their therapeutic value, their clinical translation remains challenging. The limiting factors for clinical applications of EVs include optimal culture condition establishment, along with development of protocols for large-scale EV production, isolation, and storage. Further parameters to be considered are attainment of uniformity between batches, designing optimal dosing regimens, and the development of potency assays for efficacy evaluation [290].

Largely, the lack of uniformity in EV isolation techniques, donor cell types, and experimental conditions, including cell culture conditions, makes comparisons of research outcomes difficult. Therefore, a universal EV production platform with fixed generation, isolation, and characterization protocols would be beneficial. Producer cells that can be easily genetically and/or metabolically engineered and produce high amounts of EVs are needed. MSCs and DCs are likely candidates owing to their previously demonstrated safe use in clinical trials [72].

Any potential harmful effects of the loading procedure itself should also be considered. Therefore, in comparative studies, unloaded EVs should undergo the same procedure, e.g., electroporation, as the EVs that are loaded with cargo. Even more so, control EVs could be loaded with mock cargo, e.g., non-coding DNA, scrambled control peptides, and mutant proteins.

Next, a comprehensive molecular and structural characterization of EVs and their contents is of high importance, while taking into account the variety in EV subtypes and their different functions. To empower this characterization, besides standardized isolation protocols, development of high-throughput and scalable tools to characterize molecular contents, by proteomic, transcriptomic, metabolomic, lipidomic approaches; and structural features, including biophysical properties such as size, charge, rigidity; and ultimately EV function, disease context, delivery efficiency, target cell specificity, and so on, is required (Fig. 6). This 'EV-omic' analysis could be subject to mathematical models such as principle component analysis and t-Distributed Stochastic Neighbor Embedding (tSNE) for EV subtype classification through clustering of EV characteristics. Such an approach has been recently employed based on a feature set restricted to membrane marker proteins [291]. However, a multifaceted analysis is desirable for optimal classification [291–293], assigning multiple delineating features to the different EV populations, which opens up the possibility to manipulate and design specific EV subtypes for specific applications. In addition, through the generation of searchable EV databases in conjunction with the biobanking of donor cells rather than EVs, researchers can identify important parameters needed to achieve a therapeutic function and similarly, what should be opted out to avoid unwanted effects. Development of methodologies to offload the natural cargo of EVs and substitute it for a cargo of interest, i.e., similar to the generation of empty viral capsids, may further aid in higher loading efficiency and enhanced therapeutic effects with improved safety [294]. Collective development of all of these aspects would advance the role of EVs toward realization of its candidature as a universal drug delivery carrier, as well as shed light on the mechanisms behind EV biogenesis and intercellular communication.

Author contributions

BSJ and ISZ conceptualized the review. BSJ created the illustrations. All authors wrote and edited the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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