

REVIEW

Extracellular vesicles for nucleic acid delivery: progress and prospects for safe RNA-based gene therapy

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Nucleic acid-based drugs offer a potentially effective tool for treatment of a variety of diseases, including cancer, cardiovascular diseases, neurological disorders and infectious diseases. However, clinical applications are hindered by instability of RNA molecules in the circulation and lack of efficient vectors that can deliver RNAs to target tissues and into diseased target cells. Synthetic polymer and lipids as well as virus-based vectors are among the most widely explored vehicles for RNA delivery, but clinical progress has been limited as a result of issues related to toxicity, immunogenicity and low efficiency. Most recently, the discovery that extracellular vesicles (EVs) are endogenous RNA carriers, which may display better biocompatibility and higher delivery efficiency as compared with the synthetic systems, has provided a ray of hope in coping with the delivery dilemma, and EV-based gene therapy has already sparked general interest both in academia and industry. In this review, the current knowledge on EV biology and their role in cell–cell communication will be summarized. Promises of EVs as drug carriers and recent technologies on tailoring EVs' biological attributes will be included, and preclinical studies in which EVs have shown promise for therapeutic RNA delivery will be discussed.

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INTRODUCTION

mRNAs encode proteins. *In vitro* transcribed mRNAs are emerging as a potential new drug class. These synthetic mRNAs allow transient expression of proteins, which enables a wide variety of therapeutic opportunities. mRNA-based cancer immunotherapies and infectious disease vaccines are currently clinically being explored. More challenging, however, are potential applications such as mRNA transfection to produce pluripotent stem cells, genome engineering with mRNAs encoding nucleases and *in vivo* transfection with mRNA to correct protein deficiencies.¹

Small non-coding RNAs are a class of RNA molecules with a length below 200 nucleotides that can regulate gene expression in both eukaryotes and prokaryotes.^{2,3} Non-coding small RNAs such as siRNA and miRNA offer an attractive avenue to combat various diseases by regulating the expression of genes through RNA interference (RNAi).

The achilles' heel of gene therapy is the delivery issue: naked RNA molecules cannot reach the interior of target cells because of their size, hydrophilicity and negative charge. Strategies utilizing viruses or synthetic polymers and lipids as drug carrier systems are under investigation to address these problems. However, these systems often are not desirable because of their lack of efficacy, toxicity, immunogenicity or non-specificity.⁴ For example, cationic lipids commonly used in liposome preparations are reported to invoke undesirable immune responses, whereas cationic polymers exhibit high cytotoxicity.⁵ Harmful immune responses and toxic side effects have also been reported for viral gene delivery vectors.⁶ Colloidal carriers are being recognized by the immune system as harmful particles, resulting in a hypersensitivity, or infusion reaction, recently described as complement (C) activation-related pseudoallergy (CARPA).⁷

An emerging new class of biological RNA delivery systems, that is increasingly being investigated, is known as extracellular vesicles (EVs). EVs are nanosized vesicles with a phospholipid bilayer that are released by cells in organisms from all domains, eukarya, bacteria and archaea, and are involved in intercellular communication. As EVs are released endogenously, they may offer a safer alternative for gene delivery.

Eukaryotic cell-derived EVs can be subdivided into at least three categories based on their biogenesis: exosomes (originating from multivesicular bodies (MVBs)), microvesicles (formed by direct budding of the plasma membrane) and apoptotic bodies (blebs that are formed when cells undergo apoptosis).

In Gram-negative bacteria, EVs are formed through outward budding from the surface and are known as 'outer membrane vesicles (OMVs)'. OMVs are known to have a role in intercellular as well as inter-species communication. Gram-positive bacteria also release EVs. How these vesicles have penetrated the cell wall to reach the extracellular environment is unknown and still being investigated.⁸ As little is yet known about EVs from Gram-positive bacteria, they will not be covered in this review.

The attractiveness of EVs as drug delivery systems stems from their natural ability to functionally transfer biological molecules, including genetic material.^{9,10} For example, miR-16-enriched mesenchymal stem cells-derived EVs have been shown to inhibit breast cancer cell growth by downregulating the expression of the oncogene vascular endothelial growth factor.¹¹ After proinflammatory stimuli, human blood cells have been found to secrete EVs containing miR-150, which was delivered to HMEC-1 endothelial cells and promoted their migration by silencing the c-Myb gene.¹² This capability of delivering nucleic acids implicates effective

overcoming of biological barriers, combined with a potentially superior biocompatibility.^{4,13}

This contribution will focus on eukaryotic cell-derived EVs and Gram-negative bacteria-derived EVs. First, we will discuss molecular compositions and mechanisms involved in their biogenesis, cargo sorting, intercellular trafficking and uptake. Particular emphasis is placed on the diversity of RNA species in EVs. Finally, an overview of current preclinical examples of EV-based gene therapy will be provided and current state-of-the-art technologies in engineering EV will be discussed.

MOLECULAR COMPOSITION

Proteins and lipids

The molecular composition of EVs is dependent on the EV type, the parent cell type and cell conditions, such as stress or hypoxia.^{14–16} Nevertheless, specific proteins have been shown to be enriched in (at least some subtypes of) EVs. Mammalian cell-derived EVs display high levels of tetraspanins (CD9, CD63 and CD81) on their surface, which have been implicated in regulation of the cargo loading process.^{17,18} Integrins and selectins, transmembrane receptors that mediate cell–cell and cell–matrix interactions, are also present, as well as specific lipid-binding proteins including lactadherin. These surface proteins appear to be important components in the process of EV uptake by cells.¹⁷

Besides these surface proteins, proteins which are considered to be important in the biogenesis process can also be detected in EVs, including Alix, Tsg101, clathrin and ubiquitin, as well as proteins involved in membrane trafficking. These include the family of Rab proteins and annexins.^{19,20} Antigen-presenting cell-derived EVs can also display MHC II with potential implications for immune regulatory activity.²¹

In terms of lipids, EVs are enriched in ceramides, sphingomyelin, phosphatidylserine (PS) and cholesterol compared with the parental cell membranes.⁹ A more detailed overview of EV composition, including differences between EV subtypes, is provided elsewhere.²⁰

The molecular composition of Gram-negative bacteria-derived OMVs reflects the composition of the outer membrane, which includes typical outer membrane proteins (OMPs) such as outer membrane protein A (OmpA), OmpC and OmpF, Cytolysin A (ClyA) and lipoproteins.²² Some proteins are enriched in OMVs compared with the cellular outer membrane, most notably several toxins are present at high levels in OMVs.^{23,24} The major lipid class on the outer OMV surface comprises lipopolysaccharides (LPS).²⁵ In *Pseudomonas aeruginosa*, for example, OMVs were shown to be almost exclusively composed of B-band LPS, as compared with the A-band LPS, whereas this lipid was present at much lower levels in the outer membrane.²⁶ In the OMV lumen, proteins originating from the cellular periplasmic space are primarily recovered, while levels of cytoplasmic proteins are low. The simplified molecular structure and main composition of both mammalian cell-derived EVs and Gram-negative bacteria-derived EVs are depicted in Figure 1.

RNAs

Besides proteins and lipids, RNA species are also present in EVs. The RNA species in mammalian cell-derived EVs are well studied given the advances in and accessibility of next-generation sequencing methods. These species include essentially all RNAs of the cell including mRNAs, long non-coding RNAs, miRNA, transfer RNA (t-RNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small cytoplasmic RNA, Y-RNA and vault RNA. In addition to full-length RNA molecules, some degraded products are also present in mammalian EVs. However, the relative abundance of the different classes of RNAs differs between EVs and donor cells. For example, vault RNA, SRP-RNA and Y-RNA were found to be enriched in dendritic cell-derived EVs compared with the parent cells. In addition, the presence of specific RNAs also differs between cells and EVs. Highly abundant cellular miRNAs, such as miR92a-1 and let-7b, were recovered at low levels in EVs, whereas highly abundant EV miRNAs, such as miR-223, miR-142 and miR-93, were found to be expressed at low levels in the originating cells. This suggests that

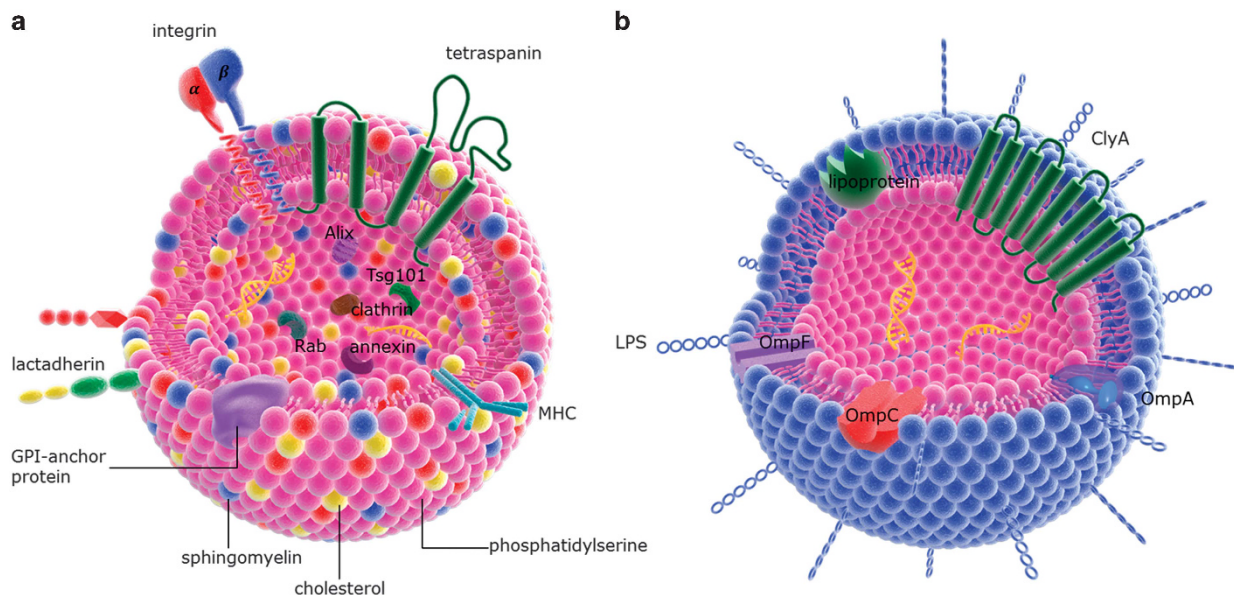


Figure 1. Simplified molecular structure and composition of EVs derived from mammalian cells (a) and Gram-negative bacteria (b). Surface protein composition of mammalian cell-derived EVs includes tetraspanins, integrins, MHC molecules, lactadherin, GPI-anchor proteins, associated with EV membrane lipids, which include sphingomyelin, cholesterol and PS. EV lumen contains RNA and proteins including Alix, Tsg101, annexins, clathrin and Rab proteins. The main composition of membranes from EV Gram-negative bacteria comprises porin proteins such as outer membrane protein A (OmpA), OmpC, and OmpF, Cytolysin A (ClyA) and lipoproteins.

RNA species are selectively packaged into EVs during their formation.²⁷

The most common RNA classes found in EVs and their enrichment in EV are summarized in Table 1. What is striking is the broad distribution of RNA classes recovered in EVs in the various studies. There are many similarities, but also some striking differences. For example, whereas Bellingham *et al.* noted that rRNA was hardly recovered from their EV samples, it was highly enriched in EVs analyzed by Nolte-^t Hoen *et al.* This could be attributed to differences in parental cell used for EV production,

but could also reflect differences in EV isolation and RNA purification or even sequencing platform.²⁸

RNA species in bacterial OMVs have been investigated to a lesser extent. It has become clear though that bacteria-derived EVs also contain a large variety of RNA species including t-RNA fragments, rRNA fragments, SRP-RNA, 6S RNA and tmRNA.²⁹ Similar to the findings for mammalian cell-derived EVs, selective packaging of specific RNA species has been noted.

EV biogenesis and cargo-sorting mechanism

EVs are generally classified according to their biogenesis. An improved understanding of the biogenesis and various actors involved can contribute to better understanding of the EV RNA cargo packaging process and help in interpreting the EV-mediated effects, and aid in designing EVs with therapeutic RNA cargoes.

Exosomes originate from precursors known as intraluminal vesicles (ILVs) formed in the lumen of MVBs in the endocytic pathway.³⁰ ILVs are formed by inward budding of the early endosome membrane. During this step, specific cytoplasmic cargo can be loaded, based on cytoplasmic abundance or affinity for the loading machinery. Upon fusion of MVBs with the plasma membrane, ILVs are released by the cell and subsequently referred to as exosomes.²⁰

The mechanism of MVB biogenesis can be broadly classified into two categories, endosomal sorting complex required for transport (ESCRT)-dependent, and ESCRT-independent. ESCRT consists of five complexes including ESCRT-0, -I, -II and -III and the associated with diverse cellular activities (AAA) ATPase vacuolar protein sorting-associated protein 4 (Vps4) complex.³¹

In the ESCRT-dependent mechanism, ESCRT-0 is responsible for sorting transmembrane proteins labeled with ubiquitin into invaginations in the endosomal membrane. This function is mainly accomplished by a phosphatidylinositol 3-phosphate-binding protein hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), which, together with signal transducing adaptor molecule (STAM), can recognize the ubiquitinated protein signal.²⁰ ESCRT-I and ESCRT-II also recognize ubiquitinated cargo and these complexes further regulate membrane bud formation. Activation of ESCRT-III subsequently leads to transient filament growth of this complex, which drives vesicle scission and recruitment of de-ubiquitinases that recycle ubiquitin. Finally, the AAA ATPase Vps4 dissociates and recycles the ESCRT machinery.³²

An alternative pathway for MVB biogenesis is ESCRT-independent. For example, through lipids such as phosphatidic acid that can induce a negative membrane curvature, or ceramide that can trigger budding of vesicles into MVBs.³³ Also tetraspanins, such as CD63, and heat shock proteins, like chaperone HSC70, can partner to induce MVB formation.³²

After formation, MVBs can either fuse with lysosomes, which leads to degradation of their content, or fuse with the plasma membrane that results in exosome release. Both RAB proteins and soluble NSF attachment protein receptor (SNARE) complexes are important in the exosome secretion process. The RAB family consists of more than 60 GTPases, some of which assist in the process of MVB fusion with the target membranes by controlling different steps of intracellular trafficking, including vesicle formation, vesicle mobility and docking to target compartments.³² SNARE proteins contribute after vesicle docking by further facilitating the fusion of MVB and plasma membrane.²⁰

Microvesicles (MVs), in contrast, are formed via direct shedding from the cell membrane and are considered to be a more heterogeneous vesicle class. Their biogenesis is less well characterized but the process seems to become activated when cells experience stress. The outward budding appears to be enabled by local membrane microdomains and curvature-induced changes in lipid and protein composition, which is complemented

Donor cell	RNA species	Enrichment in EVs	Ref.
Uninfected and prion-infected murine neuronal cells	mRNA		103
	mRNA fragment		
	miRNA	Y	
	Small nuclear RNA (snRNA)		
	Small nucleolar RNA (snoRNA)		
	Small cytoplasmic RNA (scRNA)		
	Silencing RNA (siRNA)		
	t-RNA		
	Y-RNA	Y	
	SRP-RNA (or7SL RNA)	Y	
	Long ncRNA (lncRNA)	Y	
	rRNA		
	Long intronic ncRNA		
	Piwi-interacting RNA		
	Retroviral repeats		
	Human endothelial cell line 1 (HMEC-1) cells	miRNA	
miRNA fragment			
Mitochondrial RNA (mtRNA) fragment		Y	
snoRNA			
Y-RNA fragment		Y	
mRNA fragment		Y	
lncRNA		Y	
Small Cajal body-associated RNA (scaRNA)			
7SK RNA			
Vault RNA (vRNA)		Y	
t-RNA fragment		Y	
miRNA			
t-RNA		Y	
Dendritic cells (DCs)	Y-RNA	Y	27
	rRNA	Y	
	SRP-RNA		
	SRP-RNA fragment	Y	
	Vault RNA fragment		
	Repeat		
	rRNA fragment	Y	
	snoRNA		
	snRNA		
	Mt_tRNA		
	MicroRNA		
	Non-coding RNA		
	Guide RNA		
Vault RNA			
rRNA			
RNase MRP RNA			
RNase P RNA			
Mt_rRNA			
lincRNA			
Telomerase RNA			
MDA-MB-231 cells	t-RNA fragment	Y	105
	rRNA fragment	Y	
	miRNA		
	Y-RNA fragments	Y	
MCF-7 cells	t-RNA fragment	Y	106
	rRNA fragment	Y	
	miRNA		
	Y-RNA fragments	Y	

by calcium-dependent enzymes like flippase and floppase causing redistribution of phospholipids.³⁴

Several proteins have been shown to have a role but it is unclear whether their role is cell type-specific. ARF6 has been shown to have an important role in the cargo selection and formation of MVs in melanoma cells. As ARF6 regulates extracellular signal-regulated kinases (ERK) and myosin light-chain kinase (MLCK), actin/myosin dynamics are suggested to be involved in regulating MV shedding.³⁵ Recently, RhoA with several downstream kinases was suggested as a regulator of MV release in breast cancer and cervical carcinoma cells.³⁶ In addition, a number of studies have identified calpain, an enzyme capable of cleaving cytoskeletal proteins, to be important in MV biogenesis, in particular in platelets.³⁷

The biogenesis of bacterial OMVs shows similarities to the biogenesis of MVs. Also in bacteria, OMV release is a regulated process primarily triggered by environmental stimuli, in which different stimuli induce release of OMVs of different compositions.^{38,39} The release of OMVs requires release of the outer membrane from the peptidoglycan layer. This implies that increased crosslinks between the two limits OMV release, whereas enzymes, such as endopeptidases, that degrade these crosslinks increase shedding. The reduced crosslinks cause bulging of nanodomains, which can be further promoted by accumulation of particular types of LPS and phospholipids, and specific LPS-associated molecules. Bacteria have been shown to promote shedding of OMVs in response to temperature,⁴⁰ oxidative stress⁴¹ and antibiotics.³⁸

RNA-sorting mechanisms

The abundance of a specific RNA in the cell is an important factor for its loading into EVs. Many RNA species do not appear to show preferential sorting into EVs and simply distribute over both compartments according to statistical chance. Nevertheless, specific sequence motifs appear to promote preferential EV sorting, at least in some cell types. For instance, sequence motifs including 'ACCAGCCU', 'CAGUGAGC' or 'UAAUCCCA' have been shown to serve as *cis*-acting elements in the mRNA sorting process into EVs.⁴² Similarly, mRNAs containing a zipcode-like 25-nt sequence with a 'CTGCC' core domain in addition to a miR-1289 binding site in their 3'-untranslated region are more likely to be packaged into EVs derived from human primary glioblastoma cells and melanoma cells-derived EVs.⁴³ Also, specific motifs have been

identified in miRNAs featuring 'GGAG' or 'CCCU' sequences that facilitate targeting into EVs from T-lymphoblasts, which appears to be mediated by binding to heterogeneous nuclear ribonucleoprotein A2B1.⁴⁴ These motifs for sorting miRNA into EVs have been corroborated in gamma-herpesvirus-infected lymphoma cells. Furthermore, miRNAs with 3'-end uridylated post-transcriptional modifications were found to be preferentially sorted into EVs derived from B cells.⁴⁵ This finding was validated in EVs isolated from human urine. Increasing understanding on sequence motifs that drive RNA loading into EVs may be used when trying to engineer EVs with RNAs of interest for gene therapy purposes.

For OMVs, RNA loading also seems to be regulated but the sorting mechanism has not been clearly elaborated.⁴⁶

The critical components in the biogenesis of mammalian cell-derived EVs and Gram-negative bacteria-derived OMVs are schematically depicted in Figure 2.

EV-MEDIATED NUCLEIC ACID TRANSFER IN INTRA-SPECIES, INTER-SPECIES AND INTER-KINGDOM COMMUNICATION

After release from donor cells, EVs may be taken up by recipient cells via different routes. Various reports have suggested that EVs can either directly fuse with target cells or be internalized via endocytosis.^{47–49} EVs may be endocytosed via clathrin-dependent or clathrin-independent pathways. The clathrin-independent endocytosis pathways include macropinocytosis, phagocytosis and lipid raft-mediated endocytosis.^{50,51}

Bacteria EVs can also enter host mammalian cells, through various pathways, including adhesion-receptor-mediated attachment followed by internalization and/or direct membrane fusion.⁵² EV uptake by other bacteria through direct fusion with their outer membrane has also been reported.⁵³

After internalization, EVs can mediate genetic material exchange between cells. The first reports on functional transfer of nucleic acids were shown by Ratajczak *et al.* and Valadi *et al.* Ratajczak *et al.*⁵⁴ discovered that embryonic stem cell-derived EV can transfer mRNA and protein to neighboring cells to reprogram hematopoietic progenitor cells which enhanced their survival. Valadi *et al.*⁵⁵ demonstrated that mouse mast-cell-derived EV mRNA can be transferred to human mast cells and be transcribed into protein. Subsequently, Pegtel *et al.*⁵⁶ reported that EVs derived from EBV-infected activated B cells can mediate

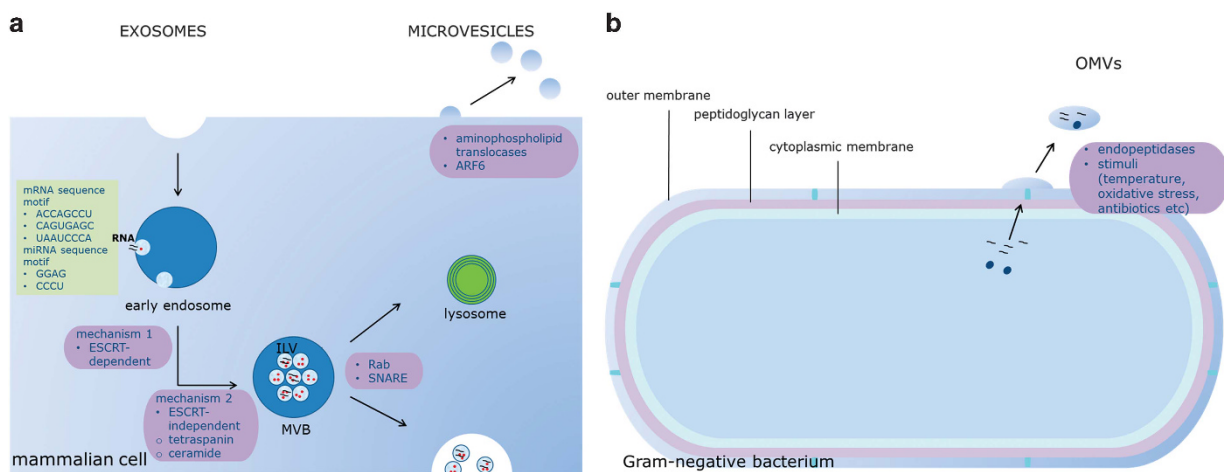


Figure 2. Biogenesis mechanism of (a) two subtypes of mammalian cell-derived EVs and (b) Gram-negative bacteria-derived OMVs. Biogenesis of mammalian cell-derived exosomes can be triggered by either ESCRT-dependent or ESCRT-independent pathways. RNAs with specific motifs may show preferential sorting into exosomes. Less is known about the biogenesis process of OMVs, however, endopeptidases and stimuli such as temperature, oxidative stress and antibiotics can contribute to OMV budding.

EBV-miRNA transfer to monocyte-derived DCs which led to downregulation of miRNA-target genes. Since then, numerous examples of functional EV-mediated RNA transfer *in vitro* have been reported, as reviewed elsewhere.⁵⁷

Most recently, several studies have used a Cre-loxP-based tracing method to provide evidence for the occurrence and significance of EV-mediated RNA transfer *in vivo*. For example, Cre mRNA carried by engineered glioma tumor cells was shown to be transferred via EVs to mouse stromal cells and subsequently lead to recombination events, as a results of translation of Cre mRNA into Cre protein.⁵⁸ In addition, this method enabled the visualization of EV-mediated Cre mRNA transfer between highly metastatic mammary MDA-MB-231 cancer cells and less metastatic T47D cancer cells *in vivo*, which was shown to change the metastatic behavior of T47D cells.⁵⁹

In the prokaryotic kingdom, OMV-mediated genetic transfer between bacteria is important in maintaining their survival. In addition, bacteria–host interactions are important in the pathogenesis of various infectious diseases.^{60,61}

An additional example of inter-species communication is that OMVs secreted by the nematode parasite can transfer miRNAs, Y-RNA and nematode Argonaute proteins to suppress the immunity response in the parasite host (mice).⁶² In addition, human cells can communicate with gut microbiota via fecal miRNA-containing EVs, which influences the bacterial gene transcript and its growth status.⁶³

The capability of EVs to transfer genetic materials makes them important players in intra-species, inter-species and inter-kingdom communication, as the genetic information exchange between donor cells and recipient cells can change the phenotype of the recipient cell.^{47,55} This newly discovered way of cell-to-cell communication is increasingly being recognized as of key importance in various physiological as well as pathological processes, as reviewed elsewhere.^{64,65}

EVS AS DRUG DELIVERY SYSTEMS FOR NUCLEIC ACIDS

Delivery of nucleic acids into target cells is notoriously difficult, especially when considering applications that require systemic administration. The large size and charged character of nucleic acids coupled to their sensitivity to degradation requires a delivery system that protects and delivers the nucleic acid to the intracellular site of activity. The most commonly studied agents for delivery are based on positively charged materials, such as lipids, and polymers, that can establish an electrostatic complex with the negatively charged nucleic acids, thereby forming nanosized particles. In such complexes, the nucleic acid is protected against serum nucleases and is formulated in such a way that allows cellular internalization.

Besides promising developments in using these synthetic systems for nucleic acid delivery, *in vitro* and *in vivo* studies have demonstrated that the positive charge of the carrier material is associated with cellular toxicity. In addition, delivery to target cells beyond the liver remains inefficient, hampering widespread use of nucleic acid technology to treat a variety of diseases. This motivates a continuing search for alternative delivery reagents.

EVs are emerging as a novel platform for nucleic acid delivery, and may be used for the delivery of both endogenous and exogenous nucleic acids. For delivery of exogenous nucleic acids, EVs have to be loaded with the desired therapeutic nucleic acid cargo first. However, loading nucleic acids into EVs is not an easy task. Nucleic acids are relatively large and charged molecules which makes it difficult or even impossible to spontaneously penetrate the EV phospholipid bilayer and to be retained inside the lumen of EVs.

Different research groups have investigated loading of nucleic acids into EVs. The majority of these studies focused on siRNA and miRNA (double stranded RNA molecules with 20–25 base pairs).

These RNA species are nucleic acids that are relatively short in length and can interfere with the function of targeted mRNAs with a complementary sequence (RNA interference). Successful delivery of these RNAs by EVs would then change the phenotype of receptor cells. The investigated loading methods can be broadly classified into two categories: loading after EV isolation and loading before EV isolation.

Loading after EV isolation

The first approach comprises loading of EVs after their isolation. In principal, such an approach may allow for loading of all EV types, independent of their cellular or intracellular origin. It is also preferred from a pharmaceutical perspective, as the loading conditions and process can be better controlled. Unfortunately, examples of successful EV loading with nucleic acids after isolation are few. Methods that have been suggested to be feasible for loading nucleic acids are discussed below.

Simple incubation. When EVs are incubated with, mainly hydrophobic, cargo of interest, the cargo may interact with the EV phospholipid bilayers driving spontaneous drug loading. Simple incubation was proven to be successful for loading of small molecules such as curcumin, doxorubicin and paclitaxel into EVs, as summarized by Tominaga *et al.*⁶⁶ Interestingly, hydrophobically modified siRNA for silencing Huntingtin mRNA was reported to be loaded into glioblastoma U87 cell-derived EVs upon incubation. In this case the modification to increase the hydrophobicity of the nucleic acids enabled them to interact with phospholipid bilayers.⁶⁷ Simple incubation has also been reported to result in the loading of miR-150 into T-cell-derived EVs.⁶⁸ This case for nucleic acid loading is quite different, as the spontaneous transport of nucleic acids over phospholipid membranes is generally regarded as negligible because the charged character of the molecule prevents efficient passage through the hydrophobic part of the bilayer. Although the mechanism behind the apparent loading remains unclear, it is likely that the miRNA is associated at the vesicle surface, which raises questions on the adequateness of miRNA protection from degrading enzymes, as well as the stability of association. It is also not clear whether the observed association is specific for miR-150 and/or T-cell-derived EVs.

Electroporation. Electroporation is a well-established method for cell transfection. Because the membrane of EVs has a similar composition as the cellular membrane, electroporation has been suggested to offer an alternative for actively introducing nucleic acids into EVs. By applying an electrical field in the medium in which EVs are suspended, small pores may temporarily form in the bilayer, which theoretically could allow entry of nucleic acid into EVs.⁶⁹

Indeed, a number of groups reported successful EV loading by electroporation. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNA and (beta-secretase 1) BACE1 siRNA appeared to be loaded into engineered dendritic cell-derived EVs by this method with high efficiency, reaching ~25% of input siRNA.⁷⁰ Similarly, opioid receptor Mu siRNA was reported to be loaded into engineered HEK-293T EVs,⁷¹ whereas mitogen-activated protein kinase 1 (MAPK1) siRNA was introduced into EVs derived from plasma.⁷² Furthermore, KSP siRNA was reported to be loaded into OMVs from a mutant *Escherichia coli* strain.⁷³ In all of these examples, EVs were shown to be capable of delivering the loaded siRNA into recipient cells, resulting in target gene knockdown. These reports also revealed that experimental parameters such as voltage, concentration of EV and cargo, electroporation medium and so on are important for the efficiency of electroporation and these parameters should be tuned to optimize the loading efficiency.

Despite these encouraging results reported for the electroporation method, Kooijmans *et al.* demonstrated that caution is warranted when interpreting results on loading efficiency. They reported evidence that, at least under certain conditions, siRNA aggregates, formed during the electroporation process, may falsely be interpreted as siRNA loaded into EVs. When this was controlled for, siRNA loading efficiencies turned out to be negligible. They also varied experimental parameters such as voltage, concentration of EVs and cargo, as well as the composition of the electroporation medium, but that did not change the overall loading efficiency.⁷⁴

It should also be noted that the EV integrity and nucleic acid structure may be affected by the electroporation procedure, demonstrating the necessity for investigating alternative approaches for EV loading.

Sonication. Active loading by sonication offers a potential novel avenue to load small RNAs without detectable RNA aggregation and degradation.⁷⁵ This sonication method may allow cargo to penetrate EV lipid bilayers by transient pore formation. MCF-7-derived EVs were reported to be successfully loaded with HER2 siRNA with this approach. However, when applying this method for EV loading, one should bear in mind that experimental parameters such as sonication power, sonication time and the temperature should be well controlled to eliminate undesirable effects on EV structure and biological activity.

Other methods. A recent report by Haney *et al.*⁷⁶ evaluated various methods for loading of the antioxidant enzyme catalase into EVs derived from macrophages, and demonstrated successful loading using different methods including EV permeabilization with saponin, freeze-thaw cycles and extrusion. However, these methods have not yet been employed for loading of nucleic acids to date. When applying these methods, one should guarantee that the structure and activity of EVs are maintained after treatment. For saponin treatment, it is also critical to ensure that remaining saponin is being removed as saponin can result in toxicity and hemolysis which may affect further experimenting.

Loading before EV isolation

The second approach, loading before EV isolation, makes use of natural EV loading mechanisms present in cells. After expression or direct transfection of target EV cargo into EV-producing cells, cargo may be sorted into EVs by the endogenous machinery of the cell, although the mechanism involved in cargo sorting remain to be elucidated.^{77,78}

Numerous reports have already demonstrated the feasibility of this approach.^{56,79} For example, HeLa and ascetic fluids-derived EVs were successfully loaded with RAD 51 and RAD 52 siRNA,⁸⁰ and Hela-229 cell-derived EVs were loaded with miR-130b by this method.⁸¹ Chemically modified miR-143 has been introduced into THP-1 macrophages, and was found to be released in EVs.⁸² In addition, viruses have been used to generate small RNA-expressing Jurkat and Raji cells, which subsequently produced small RNA-loaded EVs.⁸³

Although this method provides a straightforward way to produce EVs with desirable cargoes, several pitfalls remain. For example, transfection agents may be difficult to remove and may contaminate EV preparations. In addition, general loading efficiencies are low, dependent on cell- and cargo type, and this method may not be feasible for all RNA species (for example, expression of miRNAs that affect cell viability or proliferation may also inhibit growth of EV-donor cells).

Altogether, these reports have demonstrated proof-of-principle for the use of EVs for therapeutic RNA delivery, however, efficient cargo loading continues to form a significant hurdle for translation of nucleic acid loaded EVs to the clinic.

EV ENGINEERING FOR IMPROVING DRUG DELIVERY PROPERTIES

Although EVs seem naturally equipped to transfer nucleic acids to recipient cells, for specific therapeutic applications EVs may be engineered to improve their targeting properties, decrease immunogenicity, or to enhance functionality. For mammalian cell-derived EVs, target cell specificity seems to be largely lacking, especially after systemic administration,⁸⁴ hence improving EV targeting ability may be advantageous. The targeting properties of EVs may be increased by attaching cell-specific targeting ligands to specific molecular components on the EV surface. For example, fusion proteins of rabies viral glycoprotein (RVG) peptide, which specifically binds the acetylcholine receptor expressed on neuronal cells, and EV membrane protein Lamp2b were shown to be expressed on dendritic cell-derived EVs after overexpression in the EV-producing cells, which allowed EV delivery to the brain of mice.^{70,85} Lamp2b has also been used to display an integrin-specific iRGD (CRGDKGPDC) on EVs, which led to increased EV uptake in breast cancer cells.⁸⁶ Yet another report showed that HEK293 cells can be engineered to express the transmembrane domain of platelet-derived growth factor receptor fused to the GE11 peptide (an artificial ligand of EGFR), which subsequently produced modified EVs with GE11 displaying on their surface.^{87,88}

Besides through fusion to surface proteins, targeting moieties have also been expressed on EVs through fusion with glycosylphosphatidylinositol (GPI)-anchor peptides. With such an approach, targeting moieties would localize to GPI-rich lipid rafts in cell and EV membranes. After expression of GPI-anchored, EGFR-targeting nanobodies on EVs derived from Neuro2A cells, modified EVs presented increased binding to EGFR-overexpressing A431 tumor cells.⁸⁹

A similar approach has been utilized to enhance targeting properties of bacteria-derived EVs. Expression of genetic fusions between an anti-HER2 affibody and the C-terminus of Cytolysin A (ClyA) protein in *Escherichia coli* resulted in display of the recombinant protein on the cell surface as well as on OMVs. These modified OMVs were shown to be capable of delivering siRNA to HER2-expressing breast cancer cells.⁷³

For bacteria-derived EVs, safety issues should be considered because of the abundantly present LPS on OMVs. LPS consists of lipid A and a polysaccharide, and the latter component can hyperactivate the immune system. Hence, for drug delivery applications, approaches to reduce OMV immunogenicity may be required. The *msbB* gene that encodes acyltransferase is a well-known virulence factor. A mutation in the *msbB* gene can result in hypo-acylated LPS which displays reduced virulence.⁹⁰ For example, *E. coli* O157:H7 has been engineered to produce OMVs with mutated LPS by chromosomal tagging of FLAG-containing DNA constructs within ompA proteins, leading to mutational inactivation of the *msbB* gene. As a result, penta-acylated LPS with relatively low endotoxicity will be present on OMVs produced by mutants instead of hexa-acylated LPS as produced by normal cells.²⁵ This is analogous to *E. coli* JM83 and *E. coli* K12 W3110 strains carrying an *msbB* mutation, which are also reported to generate LPS with lower endotoxicity.⁹¹ An alternative strategy could be to shorten the o-polysaccharide group to further reduce immunogenicity.⁷³

Taken together, both mammalian cell-derived EVs and bacteria-derived EVs can be engineered to improve their targeting capacity for specific cell types, to reduce immunogenicity and to provide other functionalities, such as imaging agents. It is critical to choose an engineering site as well as subdomains of this site for consistent fusion results.⁹² It should also be noted that engineering may potentially interfere with the natural EV-cell interactions and/or affect intracellular trafficking, and negatively impact EVs' natural biocompatibility. These potential issues will have to be addressed in future studies.

EV-BASED THERAPEUTIC EFFECTS

Unmodified EVs have been used to achieve therapeutic effects. In these cases, EVs are mostly isolated from stem cells and are thought to contain a cocktail of RNAs and proteins that promote regeneration, reduce inflammation or inhibit tumor growth, as has been reviewed elsewhere.^{93,94} Such EVs have already been applied clinically. EVs derived from mesenchymal stem cells contain many of the immune-modulating factors of the parental cells. In a patient with treatment refractory graft versus host disease, treatment with these EVs reduced proinflammatory cytokines and cutaneous and mucosal symptoms of graft versus host disease and reduced diarrhea.⁹⁵ When utilized for drug delivery purposes, selecting an appropriate EV-donor cell type may therefore be of utmost importance. EVs originating from various donor cell types have different molecular compositions and thus may have different biocompatibility and immunogenic properties. Generally speaking, mammalian cell-derived EVs may have better biocompatibility as compared with the bacteria-derived EVs, due to bacteria's surface LPS composition which may trigger immune responses. But also EVs of different mammalian cell origins display distinct immunological properties. For example, although EVs derived from stem cells and immature dendritic cells harbor anti-inflammatory properties, EVs produced by mature dendritic cells carry MHC molecules on their surface and elicit

potent immune activation.⁹⁶ In addition, as EVs carry a pool of endogenous cargoes with intrinsic biological activity, their transfer to recipient cells may elicit a complex biological response, with the potential risk of introducing an undesirable phenotype. Special attention should be paid when utilizing tumor cell-derived EVs as these may carry oncogenes causative of cancer progression.

The previous chapter has illustrated that introducing exogenous nucleic acids into EVs is not straightforward but is feasible. Similarly, engineering the surface of EVs to improve targeting or delivery capabilities has been shown to be possible. This provides a toolbox to embark on therapeutic studies with tailored EVs. In this section, we provide examples of tailored EV-based therapy *in vivo* in various models of disease.

EVs from dendritic cells have been used in delivery of GAPDH siRNA, BACE1 siRNA and α -Syn siRNA specifically to mouse neural cells, which can be potentially applied in neurodegenerative disease treatment.^{70,97}

Exogenous miRNAs miR-124 and miR-145 have been introduced into MSCs via transfection, and these engineered MSCs released EVs containing miR-124 or miR-145, which induced the differentiation of neural cells and increase the expression of glutamate transporter.⁹⁸

Glioblastoma U87 cell-derived EVs could efficiently be loaded with hydrophobically modified Huntingtin-targeting siRNAs

Table 2. Overview of therapeutic applications of EV-based gene therapy

Type/source of EV	Loading method	Targeting engineering	Drug (RNA biotype)	Therapeutic effects	Ref.
Murine dendritic cells	Electroporation	Transfection with RVG-Lamp2b plasmid	GAPDH siRNA; BACE1 siRNA	Knockdown of GAPDH and BACE1	70
Murine dendritic cells	Electroporation	Transfection with RVG-Lamp2b plasmid	α -Syn siRNA	Downregulation of endogenous α -synuclein in normal mouse brain and human phospho-mimic human S129D α -Syn in transgenic mouse	97
MSCs	Transfection donor cells with miR-124 and miR-145 expression vector	—	miR-124 and miR-145	Induction of differentiation of neural cells and increase the expression of glutamate transporter	98
Glioblastoma U87 cells	Incubation EVs with hydrophobically modified siRNA	—	Huntingtin siRNA	Silencing of Huntingtin gene and protein	67
HEK-293T	Transfection of donor cells with siRNA	Transfection donor cells with RVG-Lamp2b plasmid	MOR siRNA	Downregulating MOR expression levels in mouse brain	71
Mouse fibroblast L929 cells	Transfection of donor cells with TGF- β 1 siRNA-expression vector	—	Growth factor β 1 siRNA	Suppression of S180 tumor growth in mice	100
HEK293	Transfection of donor cells with let-7a	Donor cells were transfected with pDisplay encoding GE11	let-7a	Suppression of breast tumor growth in mice	87
HEK-293T	Transfection donor cells with pCD-UPRT-EGFP	—	Suicide mRNA/protein	Inhibition of schwannoma tumor growth in mice	99
MCF-7	Sonication	—	HER2 siRNA	Knockdown of HER2 mRNA	75
Human and mouse liver cells	Transfection of donor cells with siRNA-expression vector	—	HCV siRNA; CD81 siRNA	Silencing of HCV replication and knockdown of viral entry receptor CD81	101
Human B lymphocytes	Electroporation	—	MAPK1 siRNA	Silencing of MAPK1 in monocytes and lymphocytes	72
Human plasma cells	Electroporation	—	let-7d mimics	Suppression of Th1 cells response	102
Regulatory T cells	Transfection of donor cells with let-7d mimics	—	let-7d mimics	Suppression of Th1 cells response	102
HeLa-229 cells	Transfection of donor cells with miR-130b plasmid	—	miR-130b	Inhibition of PPAR- γ Expression	81
<i>E. coli</i> K12 W3110 with msbB mutant	Electroporation	Transform <i>E. coli</i> with pGEX4T1-ClyA-affibody construct	KSP siRNA	Gene silencing and suppression of tumor growth in mice	73

(hsRNAs) upon co-incubation, and hsiRNA-containing EVs displayed target gene silencing effects after internalization by mouse primary cortical neurons, which suggests that hsiRNAs-loaded EV may be used for treatment of neurodegenerative diseases.⁶⁷

EV-based gene therapy also appears to show promising effects on treatment of drug addiction. Opioid receptor mu (MOR) siRNA could be packaged into engineered HEK-293T EVs, and these EVs were shown to efficiently deliver siRNA into neuronal cells such as Neuro2A to downregulate MOR expression, which is implicated in drug relapse but also several other central nervous system disorders.⁷¹

EV-based gene therapy has also been applied in the oncology field. Let-7 is a family of miRNAs that have been shown to act as tumor suppressors by reducing expression of oncogenes such as RAS and HMGA2. Therefore, let-7 containing EVs were explored as an antitumor agent. HEK293 cells were modified with a plasmid encoding the EGFR-targeting peptide GE11 coupled to the transmembrane domain of platelet-derived growth factor receptor. These cells were also transfected with synthetic let-7a. Overall, 1 µg of EVs injected intravenously once weekly for 4 weeks induced significant tumor growth inhibition.⁸⁷

In a similar set-up, HEK-293T cells were engineered to express high levels of the suicide gene cytosine deaminase fused to uracil phosphoribosyltransferase. EVs of these cells contained both mRNA and protein of the suicide gene. EVs were directly injected into pre-established nerve sheath tumors in mice. Upon systemic treatment with the prodrug 5-fluorocytosine, the prodrug was efficiently converted locally into the active drug by cytosine deaminase leading to tumor growth inhibition.⁹⁹ Mouse fibroblast L929 cell-derived EVs (L929 EVs) provided an efficient delivery system of exogenous TGF-β1 siRNA, which exerted inhibitory effect on the growth and metastasis of murine sarcomas 180 cells.¹⁰⁰

In addition, promising results utilizing EVs as nucleic acid vectors to treat infections,¹⁰¹ regulate immune responses¹⁰² and obesity⁸¹ have also been reported (as summarized in Table 2).

Although less well investigated, modified bacteria-derived OMVs also show promising therapeutic results by serving as drug carriers for KSP siRNA, leading to target gene silencing and highly significant tumor growth regression in a mouse breast cancer tumor model.⁷³

CONCLUSIONS

Gene therapy has the potential to be widely used in the treatment of various diseases, including cancer, infection diseases and central nervous disorders as an effective, personalized therapy approach. However, several hurdles exist when translating this potential to the clinic, including the need for delivery of nucleic acids to the diseased target site. EVs offer an attractive alternative for nucleic acid delivery from a clinical perspective, because of their biocompatibility and safety profile, as compared to more traditional polymer-, lipid- and virus-based vectors. Their versatile molecular composition makes the manipulation of their content and properties feasible. Existing challenges include identifying the right donor cells for EV production, finding an efficient way to load therapeutic nucleic acid into EVs, as well as improving the targeting properties. Efforts to address these problems will help to promote EV applications, towards safe and targeted RNA-based gene therapy in the clinic.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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