International Journal of Nanomedicine

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REVIEW

Extracellular Vesicles: A New Star for Gene Drug Delivery

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Abstract: Recently, gene therapy has become a subject of considerable research and has been widely evaluated in various disease models. Though it is considered as a stand-alone agent for COVID-19 vaccination, gene therapy is still suffering from the following drawbacks during its translation from the bench to the bedside: the high sensitivity of exogenous nucleic acids to enzymatic degradation; the severe side effects induced either by exogenous nucleic acids or components in the formulation; and the difficulty to cross the barriers before reaching the therapeutic target. Therefore, for the successful application of gene therapy, a safe and reliable transport vector is urgently needed. Extracellular vesicles (EVs) are the ideal candidate for the delivery of gene drugs owing to their low immunogenicity, good biocompatibility and low toxicity. To better understand the properties of EVs and their advantages as gene drug delivery vehicles, this review covers from the origin of EVs to the methods of EVs generation, as well as the common methods of isolation and purification in research, with their pros and cons discussed. Meanwhile, the engineering of EVs for gene drugs is also highlighted. In addition, this paper also presents the progress in the EVs-mediated delivery of microRNAs, small interfering RNAs, messenger RNAs, plasmids, and antisense oligonucleotides. We believe this review will provide a theoretical basis for the development of gene drugs.

Keywords: extracellular vesicles, gene therapy, drug delivery system, gene drugs

Introduction

In recent years, gene therapy has been widely evaluated to treat or ameliorate diseases. It can be generally defined as the transfer of gene materials to treat a disease or improve a patient's clinical condition.[1] Compared with traditional therapy, gene therapy, including gene replacement therapy, gene repair therapy and gene inhibition therapy, presents high specificity and low toxicity, and it brings a revolutionary treatment option for most genetic-related diseases.[2] Currently, many gene drugs, mainly including microRNAs (miRNAs), small interfering RNAs (siRNAs), messenger RNAs (mRNAs), circular RNAs (circRNAs), plasmid DNAs (pDNAs), antisense oligonucleotides (ASOs), CRISPR/Cas9 and DNA aptamers, have been evaluated for gene therapy.[3–7] Moreover, some gene drugs, including fomivirsen, mipomersen, defibrotide, and patisiran, have been approved by the US Food and Drug Administration (FDA) for clinical use.[8] Although these gene drugs have shown potential therapeutic effects, some limitations still exist before their translation from the bench to the bedside. For example, gene drugs can be easily degraded in vivo and they also lack active targeting, resulting in low therapeutic efficiency but high cytotoxicity for health organs.[9–11] Meanwhile, in order to improve their therapeutic efficiency, most gene drugs require the co-treatment with steroids and antihistamines which may results in the undesired side effects. Therefore, a safe and efficient delivery of gene drugs is critical for the successful gene therapy.[12,13]

To address these problems, many researchers focus on drug delivery vehicles with high transfection efficiency.[14,15] In the early years, viral vectors were the most widely used vehicles to deliver gene drugs. However, they may induce immune responses and mutagenesis in vivo.[16,17] Moreover, they can also cause various toxic and immune side effects which greatly limit their clinical applications.[18,19] In recent years, owing to their excellent biosafety and efficiency, the usage of non-viral vectors,

Received: 24 October 2023
Accepted: 20 February 2024
Published: 6 March 2024

including liposomes,[20,21] lipid nanoparticles[22,23] and extracellular vesicles (EVs),[24,25] has been an active line of research in gene therapy.

EVs as non-viral vectors have been the subject at the forefront of gene delivery in recent years.[26,27] They can inherit the properties of parent cells and obtain some components of parent cells (eg, proteins, lipids, and nucleic acids).[28] Moreover, EVs present the advantages of good biocompatibility, low toxicity,[29] low immunogenicity,[30] and easy engineering modification.[31] Additionally, EVs can protect nuclear acids and other biological macromolecules from enzymatic degradation before reaching their final destination in vivo.[32] They can also cross the blood-brain barrier,[33] and have the ability to target and deliver functional gene drugs to their destination.[34] Therefore, EVs have been widely evaluated to deliver gene drugs for the treatment of cancer, cardiovascular disease, and neurodegenerative diseases.[35–37] In this review, to promote the development of EVs as drug vehicles for delivering gene drugs, we summarize the biogenesis and composition of EVs, the generation, isolation and purification of EVs, as well as the engineering and drug-loading strategies of EVs that are presently used. Meanwhile, the challenges and prospects of EVs as gene drug vehicles are also discussed, and a scheme is presented in Figure 1.

The Biogenesis and Composition of EVs

EVs, according to their origins, are divided into exosomes and ectosomes,[38] and their secretion processes are shown in Figure 2a. In general, exosomes, with a diameter of 40–160 nm, are secreted from multivesicular bodies (MVBs) which are originated from endosomes, whereas ectosomes, including microvesicles, apoptotic bodies, and other vesicles, are released from the plasma membrane via outward budding.[39,40] Microvesicles (100–1000 nm in size), one of the major types of ectosomes, are the consequence of the extra-convexity of the cell membrane. The composition of their membrane is similar to that of the cell membrane, and the cytoplasmic components are encapsulated in microvesicles.[41] Besides that, their generation mechanisms are also different. The generation of microvesicles relies on the endosomal sorting complex required for transport (ESCRT) at the plasma membrane.[42] In the presence of ESCRT-III and actin, the plasma membrane forms protrusions and allows microvesicles to be secreted via cytosolic vomiting.[43,44] In contrast, the generation of exosomes involves both the ESCRT-dependent and ESCRT-independent pathways.[45] The ESCRT-dependent pathway in exosome generation is similar to

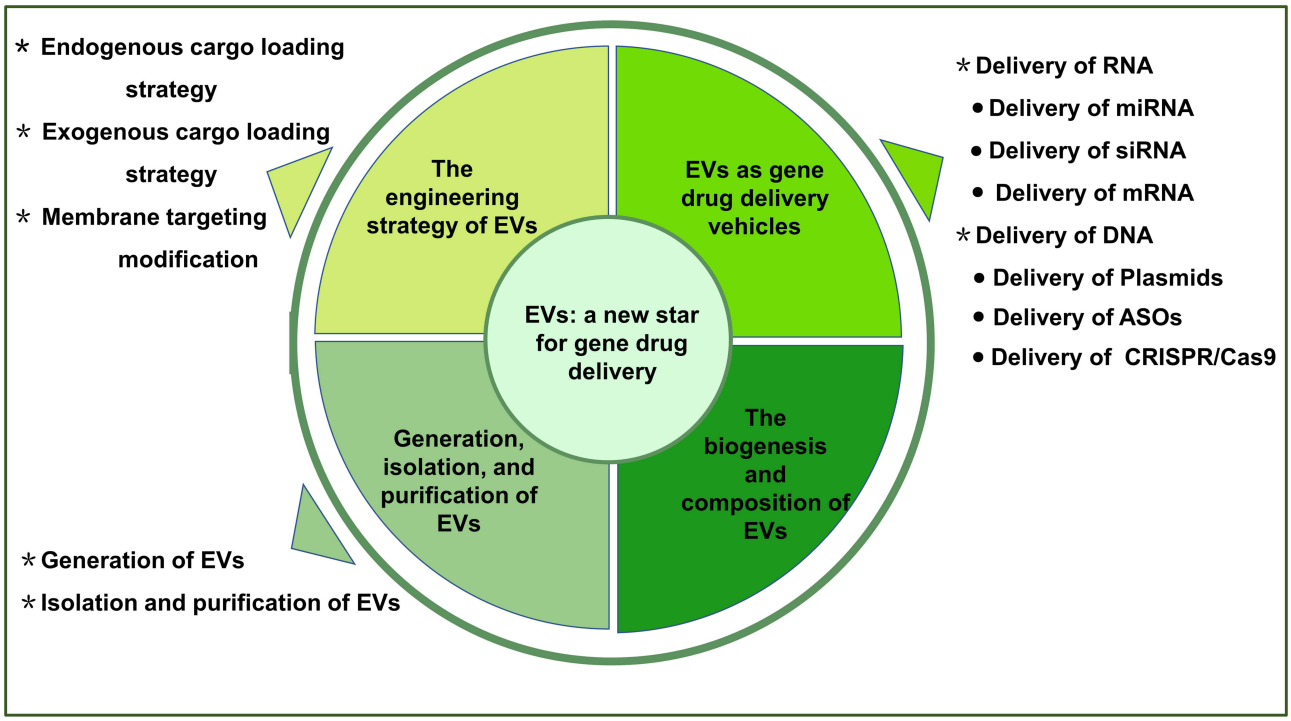

Figure 1 The main topics of this review. The main topics include the biogenesis and composition of EVs, generation, isolation and purification of EVs, the engineering strategy of EVs, EVs as drug delivery vehicles in gene therapy.

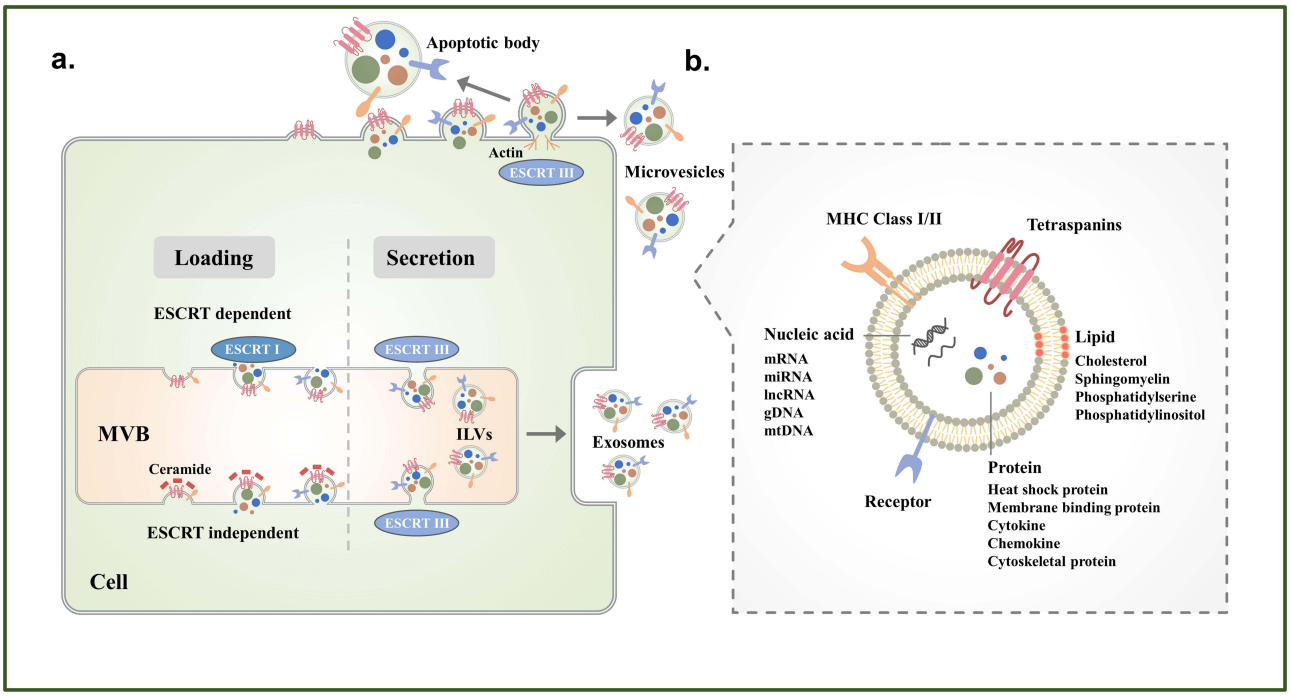

Figure 2 (**a**) The biogenesis and composition of EVs. EVs, according to their origins, are divided into exosomes and ectosomes. Exosomes, are secreted from MVBs which are originated from endosomes, while ectosomes, including microvesicles, apoptotic bodies and other vesicles, are released from the plasma membrane via outward budding. (**b**) The composition of EVs is complex, and is mainly divided into three categories: lipids, proteins, and nucleic acids.

that in microvesicles. In the ESCRT-independent pathway, cargo-loading mainly depends on sphingolipid ceramide and tetraspanins.[46] Ceramide bends the MVB membrane spontaneously, whereas tetraspanins are responsible for loading the cargo independent of ESCRT.[47,48] A more noteworthy type of ectosomes is the apoptotic body which is a special case when apoptosis occurs. Apoptotic bodies are an indicator of apoptosis and have a large distribution of diameters ranging from 50 to 5000 nm.[49] Accompanying this programmed death process, the cell membrane invaginates to wrap the intracellular components (cytoplasm, organelles, and nuclear fragments), forming multi-sized vesicles. Apoptotic bodies send the "last signal" from apoptotic cells which in turn reduce the progression of inflammatory and autoimmune diseases.[50]

The composition of EVs is complex, and is mainly divided into three categories: lipids, proteins, and nucleic acids (Figure 2b and Table 1). Since EVs are generated from membrane structures, their surfaces have the relevant components of donor cells, and abundant lipids form the basis of their structure.[51] These lipids, including phosphatidylserine, ceramide, sphingomyelin, and cholesterol, are involved in the biogenesis and release of EVs, and are highly associated with structural stability and fusion processes.[52] Therefore, the lipidomic analysis based-on EVs can provide a reference for disease diagnosis.[53] In a statement by the International Society for EVs, the EVs proteins are divided into four main categories: transmembrane or lipid-bound extracellular proteins, cytosolic proteins, intracellular proteins, and

Table 1 The Component of EVs. Molecules in EVs are Categorized into Lipids, Proteins and Nucleic Acids. Proteins Can Be Further Divided into Soluble Proteins Within Vesicles and Membrane-Bound

The Component of EVs		Ref
Lipids	Phosphatidylserine, Ceramide, Sphingomyelin, Cholesterol	[35,52,65,66]
Proteins	Cytoskeletal proteins, Rab 5/7, HSP 70/90, Oligomeric, proteins, Enzymes, Integrins, Adhesion molecules, Membrane transport proteins, Heat shock proteins, Tetraspanins, CD63/CD81/CD9, Transferrin receptor, MHC class I/II, Membrane-binding proteins	[67–70]
Nucleic acids	mtDNA, miRNA, mRNA, lncRNA, CircRNA, gDNA	[71–73]

extracellular proteins. These proteins are mainly tetraspanins (CD9, CD63, and CD81), integrins, calnexins, acetylcholinesterase.[54] These proteins are enriched during the production of EVs and are currently recognized as markers for the identification of EVs.[55–57] As another important feature of EVs, the nucleic acids such as mRNAs, miRNAs, long non-coding RNAs, and DNAs are also abundantly present in EVs.[58–60] Meanwhile, these nucleic acids in EVs also play certain roles in disease progression. For instance, miR-26b, which was highly presented in the adipocyte-derived EVs, is one of the key regulatory molecules of polycystic ovary syndrome (PCOS). Moreover, these miR-26b-containing EVs inhibited the viability of cumulus cells and promoted their apoptosis by targeting Jagged Canonical Notch Ligand 1 (JAG1). They also disrupted ovulation of the ovaries and impaired hormone levels. All these are highly associated with the high expression of miR-26b in EVs. Therefore, inhibition of miR-26b expression is expected to be a therapeutic target for PCOS.[61] Similarly, EVs isolated from human liver stem cells containing miR-145 could effectively inhibit the aggressiveness of cancer stem cells.[62] Periodontitis-damaged pulp cell-derived EVs could promote endothelial cell angiogenesis by carrying miR-378a through the down-regulation of the Sufu-activated Hedgehog/Gli1 signaling pathway.[63] Tumor mast cell-derived EVs containing miR-30a and miR-23a could inhibit osteoblast differentiation and bone mineralization in osteoporosis in patients with systemic mastocytosis.[64]

Generation, Isolation, and Purification of EVs

Generation of EVs

EVs are secreted by most, if not all, cells, and are widely presented in cell supernatants[74] and many body fluids such as urine,[75] blood,[76] and breast milk.[77] For the generation of EVs, many methods using external stimuli have been developed, as summarized in Figure 3. Typically, small amounts of EVs are released during cell culture, and EVs are obtained by a direct collection of cell culture supernatants. However, the number of secreted EVs is extremely low, making them unsuitable for large-scale experimental studies.[78] Therefore, various stimuli are applied to cells to achieve an enhanced release of EVs in large quantity.

Currently, some biological and physical methods have been used to improve the release of EVs. Biological methods mainly include cell starvation (such as hypoxia, serum starvation, and glucose starvation), genetic manipulation, and stimulation with exogenous compounds. Specifically, starvation is one of the most commonly used methods for EVs

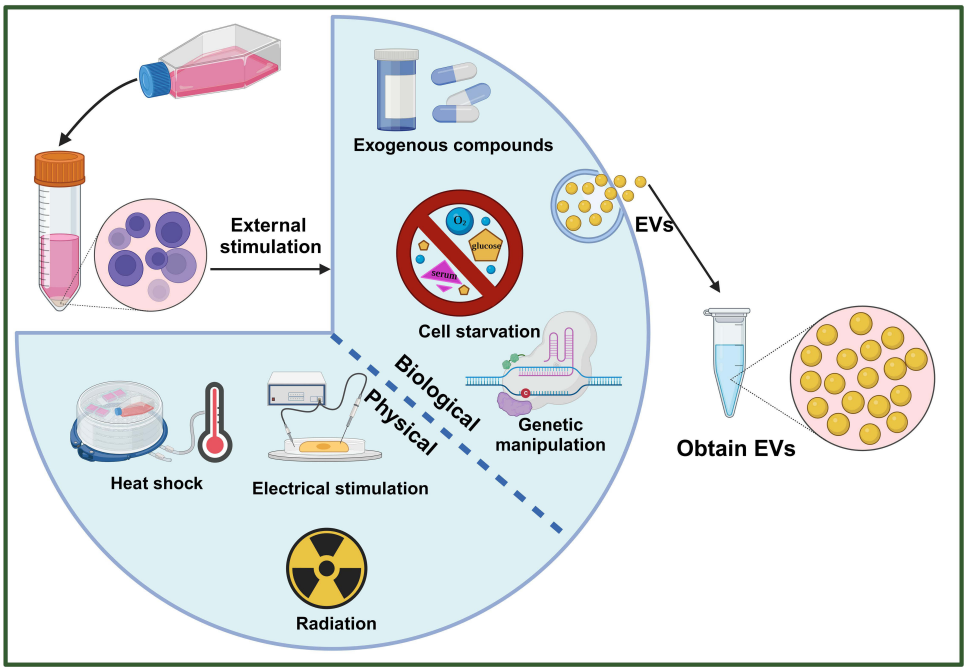

Figure 3 EVs are produced by different stimulation of the cells. External stimuli includes biological and physical methods. Biological methods include administration of cell starvation, genetic modification, exogenous compounds etc. Physical methods include heat shock, electrical stimulation, and radiation etc. Created with BioRender.com.

generation,[79] in which, hypoxia plays an important role in the cellular microenvironment and can promote the EVs production. Wang et al revealed the molecular mechanism underlying enhanced EVs production by hypoxia.[80] In general, hypoxic conditions induce the increased expression of hypoxia-inducible factor-1α (HIF-1α) that could further down-regulate the expression of ATP6V1A, which is essential for maintaining homeostasis of lysosomes. The impaired lysosomal degradation due to ATP6V1A downregulation results in reduced fusion of MVB with lysosomes and enables the secretion of intraluminal vesicles as EVs.[81] Besides the hypoxia, serum starvation method is usually used to grow cells in a basic medium with fetal bovine serum removed, and EVs are obtained by collecting the cell supernatant. Although the number of EVs obtained by serum starvation is low, it is reproducible for whole starvation and easy to perform, making it suitable for large-scale experimental studies.[82] However, some studies have shown that normal fetal bovine serum (FBS) significantly affects the biology of EVs. Therefore, during cell culture, culture medium with exosome-free FBS can be used to generate relatively pure EVs.[83] Similarly, glucose starvation method can also increase the secretion of EVs from cells. For example, the quantity of EVs released by cardiac cells was enhanced by the depletion of glucose in the cell culture medium, suggesting the importance of glucose in regulating the EVs generation process.[84] In addition to the methods aforementioned, genetic manipulation has also been used to modify the release of EVs by promoting or repressing the genes related to EVs production, and the commonly related genes in regulation the EVs generation are summarized in Table 2. Most intracellular transport pathways are determined by the conserved families of cell membrane proteins. For example, the Rab protein family controls the outgrowth, motility and fusion of vesicle. Meanwhile, they can also control the docking and fusion of different vesicle transit media to the acceptor membrane.[85] Study has shown that silencing Rab proteins can inhibit the EVs secretion.[86] Moreover, RalA and RalB in mammals are required for the production of EVs by cells.[87] In addition, Ryosuke et al found that the yield of EVs could be improved by enhancing the expression of STEAP3, syndecan-4 and a fragment of L-aspartate oxidase. Co-expression of these genes by a series of synthetic biology-inspired devices, namely EXOsomal transfer into cells (EXOtic), could greatly increase the generation of EVs. Meanwhile, EXOtic also enables the efficient and customizable production of exosomes in mammalian cells.[88] Furthermore, the intracellular components of parental cells can also be modified to enhance the EVs production. For example, the yield of EVs can be increased by the knockdown of the ESCRT protein VPS4B.[89] Additionally, exogenous substances can also increase the EVs secretion. EVs derived from mesenchymal stem cells (MSC) are also widely used in gene therapy.[90] Wang et al investigated the effects of small-molecule modulators on the secretion of EVs in MSC, and found that the combination of N-methyldopamine and norepinephrine increased the production of EVs by three folds.[91] However, the introduction of exogenous drugs into cells may induce chemical and physical damages to proteins and alter the biophysical properties of EVs, including their size, loading capacity, molecular content and cytotoxicity. Therefore, the application of exogenous substances to generate EVs is currently limited.[92]

The physical stimuli for EVs production includes heat shock, electrical stimulation, and radiation. Heat shock achieves enhanced secretion of EVs by applying heat as an external stimulus. The mechanism underlying the secretion of EVs in response to heat shock in cancer cells was evaluated by Otsuka et al. They found that the growth of cancer cells and the secretion of EVs were increased in a temperature-dependent manner by the low-density lipoprotein receptor (LDLR), a gene which was upregulated with increased temperature.[93] Different from heat shock stimulus, the release of EVs from electrically stimulated cells can be continuously similar to the external environment because cells continuously repair themselves after electric shock damage, providing a possibility for the mass production of EVs.[94] Zhang et al demonstrated that electrical stimulation method significantly increased the release of cardiac MSC-derived EVs, and the EVs obtained through a neutral sphingomyelinase 2 (nSMase2)-dependent mechanism had an enhanced cardioprotective effect in C-MSCs.[95,96] Our group has also developed a cellular nanoporation (CNP) technology[33] as shown in Figure 4. In this work, cells were stimulated by

Table 2 The Type of Gene Manipulation Affects EVs Generation

Gene Type	Effectiveness	Production of EVs	Ref
Rab2b, Rab9a, Rab5a, Rab27a, Rab27b, RalA, RalB	Low	Low	[86,87]
STEAP3, syndecan-4, and a fragment of l-aspartate oxidase	High	High	[88]
VSP4B	Low	High	[89]

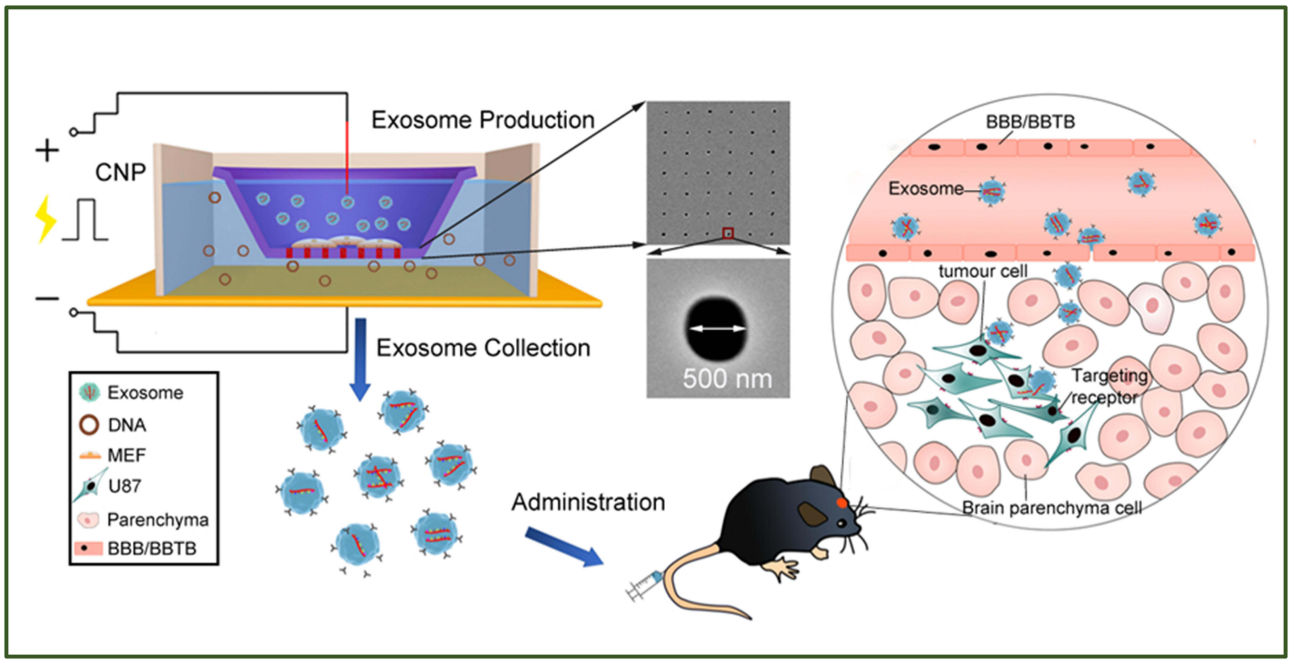

Figure 4 Schematic representation of CNP-generated EVs for targeting nucleic acid delivery.
Notes: Reprinted from Yang ZG, Shi JF, Xie J, et al. Large-scale generation of functional mRNA-encapsulating exosomes via cellular nanoporation. *Nat Biomed Eng*. 2020;4(1):69–83.[33] Copyright © 2019, The Author(s), under exclusive licence to Springer Nature Limited.

transient electrical pulses to generate nanopores on the cell membrane, which in turn facilitated the loading of pDNA from the buffer into the cells. The experimental results showed that CNP not only improved the efficiency of gene delivery, but also produced more than 50 times of EVs as compared to the conventional methods. The transient upregulation of calcium ions inside the cells through the pores generated by CNP in the first 5 mins and the extremely high temperature near the nanopore are responsible for the massive EVs generation. Compared with other physical methods, it is a prospective method for EVs loading and generation in a single step. In addition, radiation, including X-ray,[97] gamma ray,[98] and ultraviolet,[99] has been shown to increase the secretion of EVs in various cells. However, the application of radiation can cause cell damage and affect the experimental results, such as the production of reactive oxygen species under ultraviolet irradiation which can induce oxidative cell damage if its level exceeds the antioxidant defense mechanism of the body. Therefore, the application of radiation for the large-scale production and application of EVs is still controversial, though it has some advantages over other methods discussed above.[100]

Isolation and Purification of EVs

For the isolation and purification of EVs, differential centrifugation, density gradient centrifugation, immunoseparation, polymerization precipitation, microfluidic chip separation, tangential flow ultrafiltration, and size-exclusion chromatography (SEC) are the methods widely used (Figure 5). [101] Each method has its own advantages and disadvantages, which are summarized in Table 3.

Among all the methods developed, differential centrifugation is the most widely used for the isolation and purification of EVs because it can obtain relatively pure EVs by removing cells and debris at low-speed centrifugation and then precipitating the EVs by ultracentrifugation. However, the major problem with this method is its extremely low yield of EVs. Depending on the procedure, only approximately 10% of the total EVs can be enriched from the sample. However, using this method, almost no chemical contamination of EVs is achieved since only simple agents are used in the ultracentrifugation steps. This is critical when the EVs are used in vivo since no immunogenicity or side effects can be induced. Unfortunately, when it comes to biofluids, EVs cannot be completely separated from high-density lipoproteins in blood plasma or serum, resulting in the low purity of the final products.[110] Although many studies have addressed this

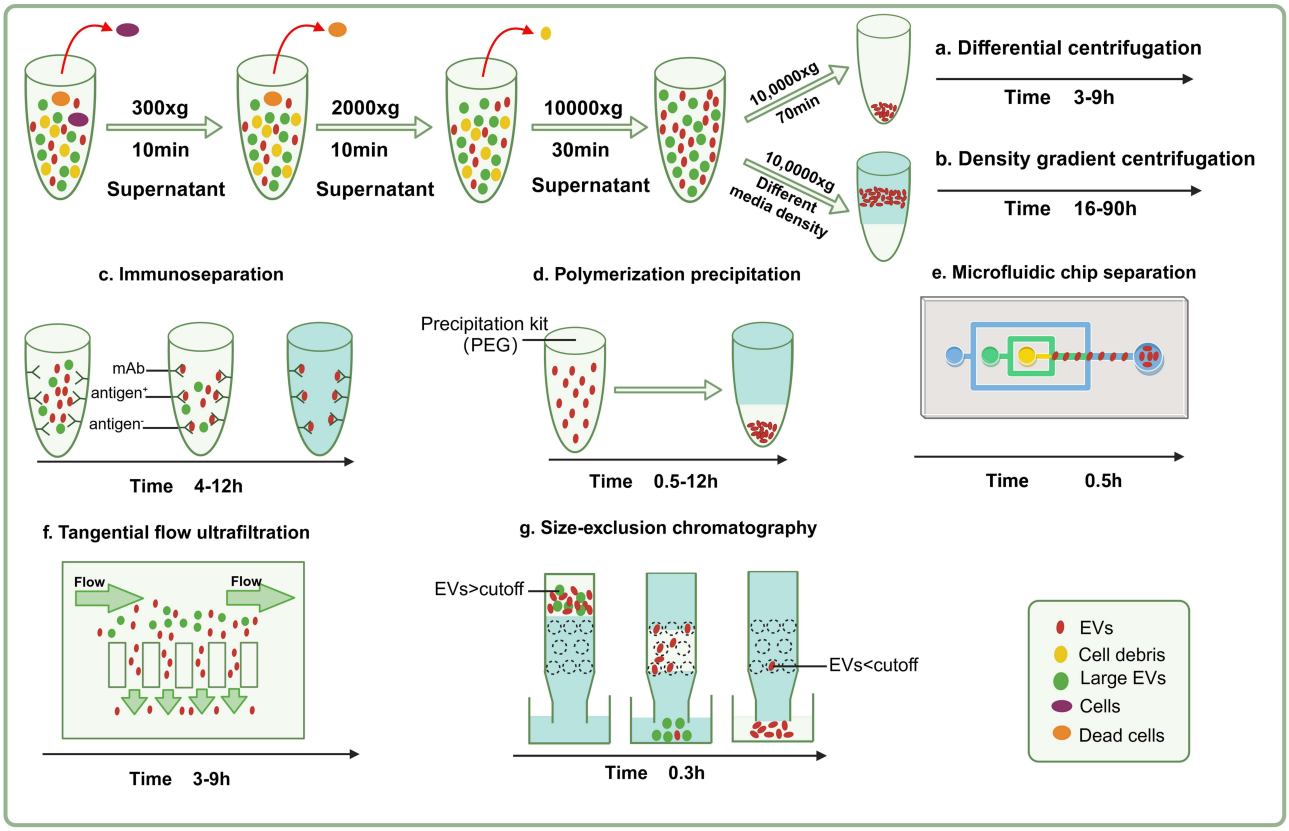

Figure 5 The isolation and purification methods for EVs. (**a**) Differential centrifugation: separating EVs based on differences in centrifugal speeds. (**b**) Density gradient centrifugation: the sample is first centrifuged at differential speed, and EVs are finally separated based on the densities of different media. (**c**) Immunoseparation: it achieves EVs' separation by selectively binding to antigen receptors on the EVs membrane. (**d**) Polymerization precipitation: it precipitates EVs from the target sample through polymers. (**e**) Microfluidic chip separation: it is a technique in which physical techniques are used to capture EVs in microfluidics by nanomaterials or chips. (**f**) Tangential flow ultrafiltration: it separates EVs in the form of centrifugal filtration based on molecular weight cut-off. (**g**) SEC: it separates EVs based on the size of the sample. Created with BioRender.com.

difficulty by the combination of other methods or additional steps with ultracentrifugation, the final yield of EVs is further compromised.[102] Density gradient centrifugation is another commonly used method for EVs isolation. Its principle is based on the buoyant density, sedimentation coefficient, and particle size of the separated substance. Substances with similar particle sizes and buoyancy densities can be obtained using this method. However, one

Table 3 The Advantages and Disadvantages of EVs' Isolated and Purified Method

Method	Advantages	Disadvantages	Ref
Differential centrifugation	Simple operation and high collection rate	Long time consumption, expensive instruments, damage to the integrity of EVs	[102,103]
Density gradient centrifugation	The purity of the separated fractions can be improved	Time-consuming	[104]
Immunoseparation	High specificity and purity	Only enrichment of EVs with specific proteins	[105]
Polymerization precipitation	High production and low time consumption	Low purity, low activity of EVs	[106]
Microfluidic chip separation	Automation, low consumption time	Only suitable for small volume samples, high technical level	[107]
Tangential flow ultrafiltration	Simple operation, high efficiency, suitable for large volume	Residual lipoprotein and chymotrypsin	[108]
Size-exclusion chromatography	Mild, low damage, high quality and purity	Costly equipment	[109]

shortcoming of this method is that it is almost impossible to separate a specific group of EVs of interest from the mixture. Due to their different origins, EVs have great overlaps in their sizes and densities. Even within the same group of EVs, such as exosomes, they may contain different surface biomarkers. Therefore, the purity of the EVs after ultracentrifugation is dampened. Moreover, this method is inconvenient since it requires a high-speed ultracentrifugation for a relatively long time (typically greater than 10 hours for ultracentrifugation).

Originated from traditional ultracentrifugation method, many new methods have been innovated, each with its own pros and cons. The immune separation method, also known as the immunoaffinity capture method, has the advantage of enriching certain classes of EVs with high specificity.[105] However, it can only enrich the expression of one or several specific surface proteins for EVs and thus has a relatively limited range of applications.[111] Polymerization precipitation is relatively convenient compared to other methods since commercial kits are typically used for EVs precipitation. However, it introduces numerous chemical reagents (polyethylene glycol)[112] and EVs obtained by polymerization-precipitation are often mixed with impurities such as proteins and polymers, which unfortunately leads to a decrease in the purity of EVs. Meanwhile, virus particles in the sample can also be co-precipitated, which will affect the downstream analysis (proteomics and mass spectrometry).[106] The microfluidic chip separation method is a technique in which physical techniques are used to capture EVs in microfluidics by nanomaterials or chips.[107,113] For example, researchers generated a CD63-based immunoaffinity microfluidic device based on the characteristics of EVs owing to their surface-specific proteins and lipids, such as CD63.[114] However, due to the low expression of CD63 in EVs secreted by some cancer cells, anti-CD63 was not specific for EVs of cancer cell origin. In order to overcome this drawback, Kang et al designed a new exosome isolation microfluidic device to capture EVs based on the overexpression of phosphatidylserine on EVs surface, achieving 90% capture efficiency for EVs with cancer cell origin.[115] Currently, more researches are being conducted to continuously optimize the conditions for capturing EVs step by step according to the various expression levels of different proteins on the surface of EVs to achieve a high yield of EVs. Although the microfluidic chip separation method can enrich EVs efficiently and specifically, it is suffering from high-level technological requirements and is only suitable for the separation of EVs within small volume of samples. In comparison, the tangential flow ultrafast method is the easiest and fastest method that does not require expensive instruments and is suitable for the separation of EVs from large volumes of samples such as serum. Owing to these advantages, tangential flow filtration-based EVs preparation has been applied to isolate EVs in various clinical trials.[116,117] However, one of its disadvantages is that it is easy to retain substances such as lipoproteins and chymotrypsin.[118] In addition to these methods, SEC is becoming increasingly popular and is a method of purification based on the difference in the particle size of EVs which allows the recovery of pure vesicles with higher functionality, purity, and integrity. The advantages of SEC include the reduction of EVs aggregation during separation, the effective separation of EVs from soluble proteins, and the ability to isolate pure, intact, and biologically active EVs.[119] Therefore, this method has become increasingly popular for EVs separation and purification in recent years. Although an enhanced purity and efficiency was demonstrated, it came with a cost of further dilution of the final products. Therefore, an additional concentration step may be required in subsequent experiments.

Although various methods have been developed for the isolation and purification of EVs, the large-scale handling of EVs remains challenging. Moreover, the purity and yield of the general product cannot be guaranteed which would have an impact on the quality of EVs.[120] The main reason for this is that various subgroups of EVs overlap with the physicochemical properties, sizes of proteins, lipoproteins, and abdominal particles.[121] However, some of the methods discussed above can attenuate these difficulties though they may also create new dilemmas; therefore, researchers should choose the appropriate extraction and isolation method according to their experimental needs.

The Engineering Strategy of EVs

Natural EVs, when used as drug carriers, are difficult to target to the disease site and accumulate in the organs, resulting in low drug availability.[65,122,123] Therefore, proper engineering modifications of EVs can increase their delivery efficiency, targeting ability, and therapeutic efficacy.[124–127] In general, the engineering strategy of EVs mainly includes cargo loading, such as endogenous and exogenous cargo, as well as membrane-targeting modification, as shown in Figure 6.

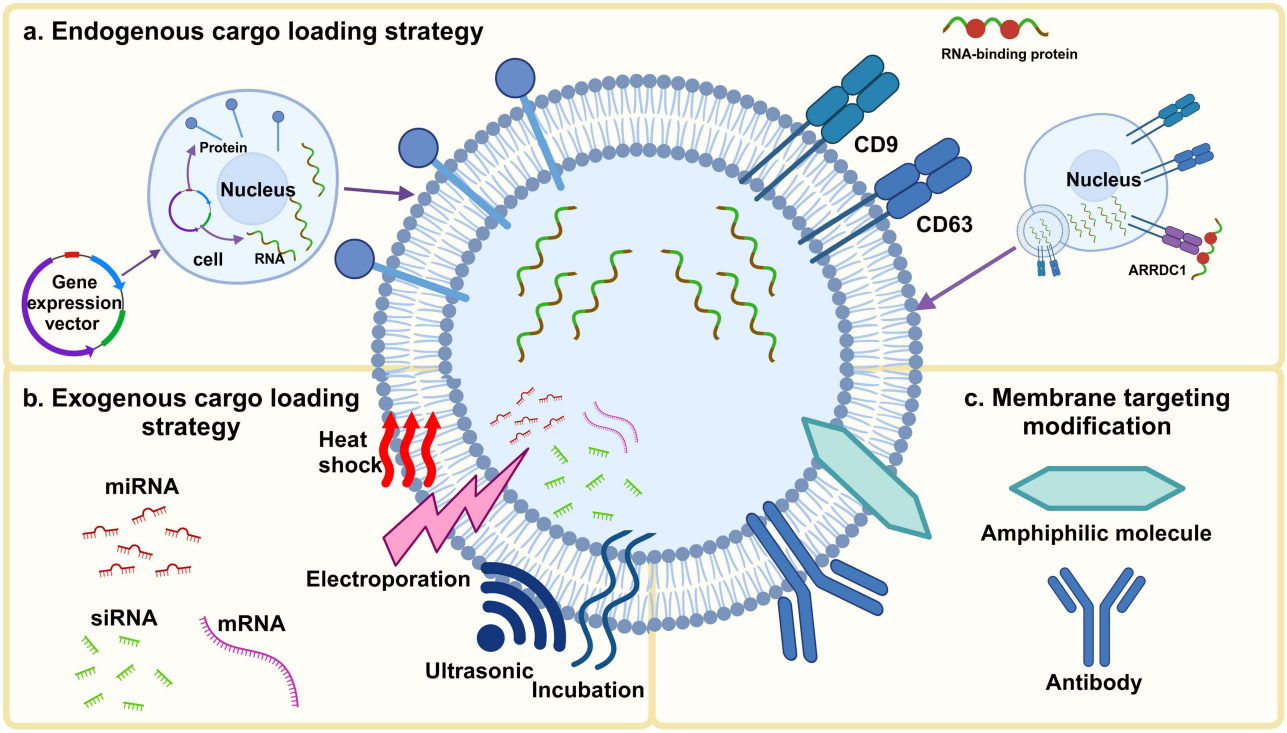

Figure 6 The engineering strategies for EVs. (**a**) Endogenous cargo loading strategy: it generates functional EVs by the endogenously loading of functional molecules into cells. (**b**) Exogenous cargo loading strategy: it utilizes loading strategies such as incubation, electric transfer, heat shock, ultrasound, etc. to directly load exogenous goods into EVs, thereby exerting the effect of exogenous goods. (**c**) Membrane targeting modification: it enhances the targeting ability of EVs by binding functional molecules to proteins or phospholipids on the surface of EVs. Created with BioRender.com.

Endogenous Cargo Loading Strategy for the Engineering of EVs

Cell engineering is a promising method for introducing functional molecules into donor cells.[128,129] EVs can obtain these properties by utilizing the ability to inherit properties from their parent cells.[130] Plasmids and viral vectors are typically used for endogenous cargo loading into EVs. They were loaded into the donor cells, after which the target genes were expressed in the donor cells. Meanwhile, the desired molecules can be loaded into EVs before being secreted into the extracellular medium.[30,131]

Transfection, which is the most commonly used method, introduces target genes into specific cells using plasmids or viral vectors.[132] In which, electroporation, as one of the commonly used transfection methods, can disrupt the cell membrane and increase its permeability to improve the loading efficiency of exogenous substances under an instantaneous and strong electric field. This method not only stabilizes the transfected cells but also improves their transfection efficiency. For example, Zhao et al transfected left-right determination factor 1 (lefty1) mRNA plasmids into cells to generate engineered EVs loaded with lefty1 mRNA by electroporation, and these EVs could effectively attenuate liver fibrosis.[133] However, low secretion efficiency of EVs was demonstrated by this conventional electroporation. In order to overcome this drawback, our group designed a CNP to load DNA plasmids into cells.[33] Compared with conventional electroporation, more than 50 times of EVs were secreted using this method and showed a high therapeutic effect in glioma.

In addition, protein fusion methods can also be used to engineer EVs. Researches showed that tetraspanins (CD9 and CD63) which contain extracellular and intracellular domains are highly enriched on the surface of cells, and endogenous substances can be loaded by utilizing these proteins. For instance, Es-Haghi et al[134] developed a fusion protein of EVs membrane protein CD9 and RNA-binding protein AGO2 (hCD9.hAGO2) to achieve the RNA loading, and the results indicated that EVs contained high levels of miRNA and these engineered EVs could also transfer RNA cargo to recipient cells more efficiently. Bui et al[135] achieved endogenous cargo loading by interacting with CD63 and improved the delivery efficiency of EVs by fusion without requiring any viral fusogenic protein. The results indicated that the delivery efficiency of the cargo was significantly improved by the overexpressing of Syncytin-1. In addition, arrestin domain

containing protein 1 (ARRDC1) is also an important protein to carry specific molecules into microvesicles. It can recruit the TSG101 protein to the plasma membrane and drive plasma membrane outgrowth to release microvesicles.[136] Therefore, the binding between ARRDC1 and endogenous cargo can be exploited for the engineering of EVs. Wang et al[137] designed and constructed ARRDC1 mediated EVs for the delivery of P53 protein, RNAs, and CRISPR/Cas9/guide RNA, and results showed a high loading efficiency for endogenous cargo. Although these methods for engineering EVs presented excellent results, high loading and delivery efficiency still need to be explored.

Exogenous Cargo Loading Strategy for the Engineering of EVs

Exogenous cargos including a variety of therapeutic agents can be directly loaded into isolated EVs by membrane penetration or other loading strategies. Compared with the endogenous cargo loading strategy, strategies for loading exogenous cargo are simple and have been widely used to develop novel drug delivery systems. Multiple strategies, including incubation, electroporation, heat shock, and ultrasound, have been used to modify EVs, and the advantages and disadvantages of these methods are summarized in Table 4. [138,139] Incubation, which uses a drug concentration gradient to get external drugs into EVs, is the simplest and fastest method of gene drug loading though it is suffering from its extremely low drug-loading capacity.[140] In contrast, electroporation is one of the most commonly used gene drug-loading methods with relatively high encapsulation efficiency, as compared with the incubation method.[141] Studies found that electroporation achieved a high drug-loading efficiency and a low amount of protein damage to the surface of EVs.[142] Nucleic acids and other macromolecules can be electrotransferred into EVs to perform their functions. However, the traditional electroporation also has some limitations. For instance, the aggregation of insoluble siRNA may occur, leading to a relatively low encapsulation efficiency.[143] In addition, the heat shock transfection method is also one of the commonly used methods. Zhang et al enriched miRNAs into EVs using a modified calcium chloride-mediated heat shock transfection method, and demonstrated that miRNA mimics or inhibitors delivered into recipient cells by EVs could cause efficient overexpression or deletion of miRNAs in recipient cells.[144] Meanwhile, studies have shown that ultrasound can also be used for the loading of small RNA into EVs,[145] and it can avoid the aggregation or degradation of therapeutic RNA cargo.[146,147]

Membrane Targeting Modification

For the membrane modification of EVs, it is a way to enhance the targeting of EVs to receptor cells. Since the surface of EVs is similar to that of cell membranes, and the main component is the lipid bilayer, it also carries abundant transmembrane and extracellular proteins, as well as amphiphilic phospholipids. Therefore, the principle of EVs surface membrane modification is to functionalize EVs membrane by combining functional molecules with proteins or phospholipids on the EVs surface. For example, amphiphilic phosphatidylcholine (PC) molecules can be inserted into the membrane surface of reticulocyte-derived exosomes. Compared with unmodified exosomes, PC-modified exosomes showed a two-fold increase in tumor cell internalization efficiency and uptake rate.[149] In addition, the EVs surface can be modified with target antibodies by simple incubation with commercial antibodies to achieve targeting, and its efficiency depends on the number of proteins on the EVs surface that can bind to targeting antibodies.[150] Moreover, EVs mimicking nanovesicles are similar in morphology, size, and

Table 4 The Advantage and Disadvantage of Exogenous Cargo Loading Strategy

Type	Advantages	Disadvantages	Ref
Incubation	This approach is simple to perform and does not affect the integrity of the EVs	It has the disadvantage of being less efficient in loading and more effective for hydrophobic molecules and exporting low drug concentrations	[132]
Electroporation	The method can be loaded with hydrophobic and hydrophilic compounds	This method impairs membrane integrity and tends to cause aggregation or fusion of EVs	[141]
Heat shock	Ensure stable protein expression	Low loading efficiency	[144]
Ultrasound	This method is relatively efficient and can be loaded with both hydrophilic and hydrophobic compounds	Disrupts membrane integrity and alters EVs' size	[148]

function, compared with natural EVs. They are developed by the extrusion of cells, and their yield is higher than that of natural EVs. Among all the methods for EVs mimicking nano-vesicles generation, extrusion of the generated EVs mimicking nanovesicles on polycarbonate membrane filters is the most common and simple method.[151] In addition, the method of generating EVs mimicking nanovesicles has been further optimized to obtain large quantities of EVs,[152] such as the extrusion of cells using centrifugal force with microporous channels,[153] and applying 500 nm thick silicon nitride (SixNy) blade to cut live cell membranes to produce nanovacuoles.[154] In addition, some methods, including gene modification, lipid insertion, metabolic modification, and affinity binding, have been applied to membrane-targeted modifications of EVs.[67] Therefore, EVs are endowed with some characteristics that give EVs a greater ability to target or inhibit disease, as shown in Table 5.

In conclusion, engineered EVs can overcome the problems of low yield, low loading efficiency, and low targeting. Further loading of engineered EVs with gene drugs promotes the maximum therapeutic effect of gene drugs at disease sites and enables safe drug delivery.

EVs as Gene Drug Delivery Vehicles

EVs present the advantages of good biocompatibility, low toxicity, low immunogenicity, and easy engineering modification. Moreover, EVs can protect nuclear acids and other biological macromolecules from enzymatic degradation before reaching their final destination in vivo. More importantly, autologous EVs are easily obtained from blood or other body fluids, which makes them safe and induces low side effects. Consequently, EVs have become an excellent deliver vector for gene drugs. In this section, we mainly discuss the application of EVs in RNA delivery, including the delivery of miRNAs, siRNAs, and mRNAs, as well as their application in the delivery of plasmids, ASOs, and CRISPR/Cas9.

Delivery of RNAs by EVs

RNAs, which are commonly found in animals, plants, microorganisms, viruses, and phages, are a class of nucleic acids consisting of at least a few dozen ribonucleotides linked by phosphodiester bonds, and are intimately related to protein biosynthesis.[160] Therefore, they play key roles in biosynthesis, regulation, and intercellular signaling in vivo.[161] At present,

Table 5 The Example of Membrane-Targeted Modification of EVs and Its Application, the Main Modalities Include Gene Modification, Lipid Insertion, Metabolic Modification, and Affinity Binding

Modification Methods	Modification Principle	Targeting molecule	Application	Ref
Gene modification	Many membrane proteins are highly expressed on the surface of EVs, such as CD63, CD81, CD9, and Lamp2b. Genes were fused to these membrane proteins and used to display targeted fragments on EVs.	Lamp2b	Lamp2b-guided neurotargeting peptide RVG conducts *BACE 1* siRNA for Alzheimer's disease.	[155]
		CD64	This study prepared EVs overexpressing CD64 protein on the surface, which actively recruits IFN-γ mRNA within itself.Specific mRNA loading and tumor targeting were achieved.	[131]
Lipid insertion	EVs membranes can be hydrophobically inserted by lipids and lipid-labeled molecules to enhance targeting.	DSPE-PEG-RGD	Combined enhancement of vascular targeting and angiogenesis.	[156]
		Cholesterol-AS1411 aptamer	Enhanced delivery of targeted breast cancer to inhibit tumor growth.	[157]
Metabolic modification	Donor cells were cultured in medium containing azide-containing amino acids or azide-containing sugars, which were metabolically introduced to the surface of the EV membrane.	Azadibenzylcyclooctyne-fluorescent dyes	Uptake labeling applied to EVs	[158]
Affinity binding	Molecules with targeted effects work by attaching to affinity molecules of EV membrane proteins or lipids.	Superparamagnetic nanoparticles	Enhanced in vivo targeting effect and cancer inhibition	[159]

RNA drugs primarily include miRNAs, siRNAs, mRNAs, RNA decoys, and circRNAs.[162] Although they present good therapeutic effects, they also have many problems, such as easy degradation, low therapeutic efficiency, and non-selectivity.[8] Therefore, there is an urgent need to identify a vector that can safely and efficiently deliver gene drugs to cells or tissues. EVs serve as a novel drug delivery system with the ability to protect nucleic acids from degradation in vivo[163] and avoid attacks from the immune system, thus ensuring the effective targeting of RNA into target cells.[5] In this section, because there are few studies on RNA decoys and circRNAs delivered by EVs, we focus on summarizing miRNAs, siRNAs, and mRNAs.

miRNA

miRNAs are a class of small endogenous non-coding RNAs of 20–25 nucleotides with regulatory functions. miRNAs have many functions such as inhibiting target mRNA transcription and translation, shearing target mRNA, and promoting its degradation.[164] The regulatory role of miRNAs is involved in tumor progression, invasion, angiogenesis, and metastasis.[165] Studies have shown that miRNA expression is dysregulated in cancers and other diseases.[166] Therefore, altering the miRNA levels in target cells has the potential to serve as a therapeutic intervention.[165] miRNA wrapped in EVs for systemic drug delivery can well-solve currently existing problems facing the miRNA delivery field (such as low miRNA targeting, high toxicity, poor delivery, and rapid degradation).[167,168] Moreover, EVs-miRNA can stably reach the disease sites and regulate the expression of downstream genes.[169] Ohno et al demonstrated that EVs loaded with let-7a miRNA bound to an epidermal growth factor (EGF)-specific peptide (GE11) can be delivered to EGFR-expressing breast cancer cells with high therapeutic efficiency.[170] In another study, the authors identified two endogenous miRNAs (miR-155 and miR-146) abundant inside dendritic cell-derived EVs that can mediate target gene repression, and the results indicated that miR-146a delivered by EVs could dramatically decrease inflammatory gene expression.[171] In addition, by delivering the miR-181c as the payload inside, cancer cell-derived EVs can downregulate the target gene PDPK1, which in turn leads to abnormal localization of actin, resulting in the degradation of the blood-brain barrier.[172]

siRNA

siRNAs are a class of double-stranded RNA molecules with 20–25 base pairs in length, and are involved in the RNA interference (RNAi) process.[173] They can block the protein translation by interfering with the mRNA transcription. Therefore, siRNAs are of great research value for the silencing of disease-related genes. Several studies have indicated that the delivery of siRNA via lipid nanoparticles and other carriers may cause toxicity due to the high dosage requirements for patients. Therefore, many clinical trials using lipid nanoparticles for siRNA delivery have been terminated.[174] Recently, studies have shown that EVs are ideal carriers for the delivery of siRNA to achieve the knockdown of target gene.[175,176] Compared with artificial nanomaterials used in lipid nanoparticles, the application of EVs for siRNA delivery is a simple and effective low-risk treatment strategy. Currently, however, the bottleneck in the application of EVs for siRNA delivery lies in that the loading rate of siRNA into EVs is greatly limited. Therefore, the modification and improvement of the siRNA loading method into EVs are of great value. In a recent study, to better load siRNA into EVs, Diao et al developed a novel strategy to encapsulate siRNA into EVs using the polycationic membrane-penetrating peptide TAT. By their new method, the limitation of the siRNA loading rate was greatly improved, and the engineered EVs could effectively downregulate androgen receptor (AR) expression and induce LNCaP AI cell apoptosis.[177] In another study, siRNA was integrated into the pre-miR-451-derived RNA backbone, and it was found that EVs could package the target siRNA with high efficiency. Compared with lipid nanoparticles, EVs showed a better delivery efficiency since they only require 10 times less siRNA to silence the target gene, and this could effectively reduce the cytotoxicity induced by siRNA.[178] Meanwhile, high siRNA transfection rates could also be achieved by engineering the membranes of EVs. For example, by separating tumor-derived EVs (TDEV) from hepatocellular carcinoma (HCC) cells (abandoning their contents) and fusing them with phospholipids to construct TDEV membrane-hybrid lipid nanovesicles (LEVs), this delivery platform could carry specific siRNA to precisely reach tumor sites and achieve target gene silencing in a mouse liver cancer model (Figure 7a).[25] In addition, Wang et al used the aptamer AS1411 (a DNA aptamer that binds to nucleolin with high affinity and is overexpressed on breast cancer cell plasma membranes)-modified EVs to bind nucleolin, and this strategy could target-deliver siRNA/miRNA to breast cancer tissues.[157]

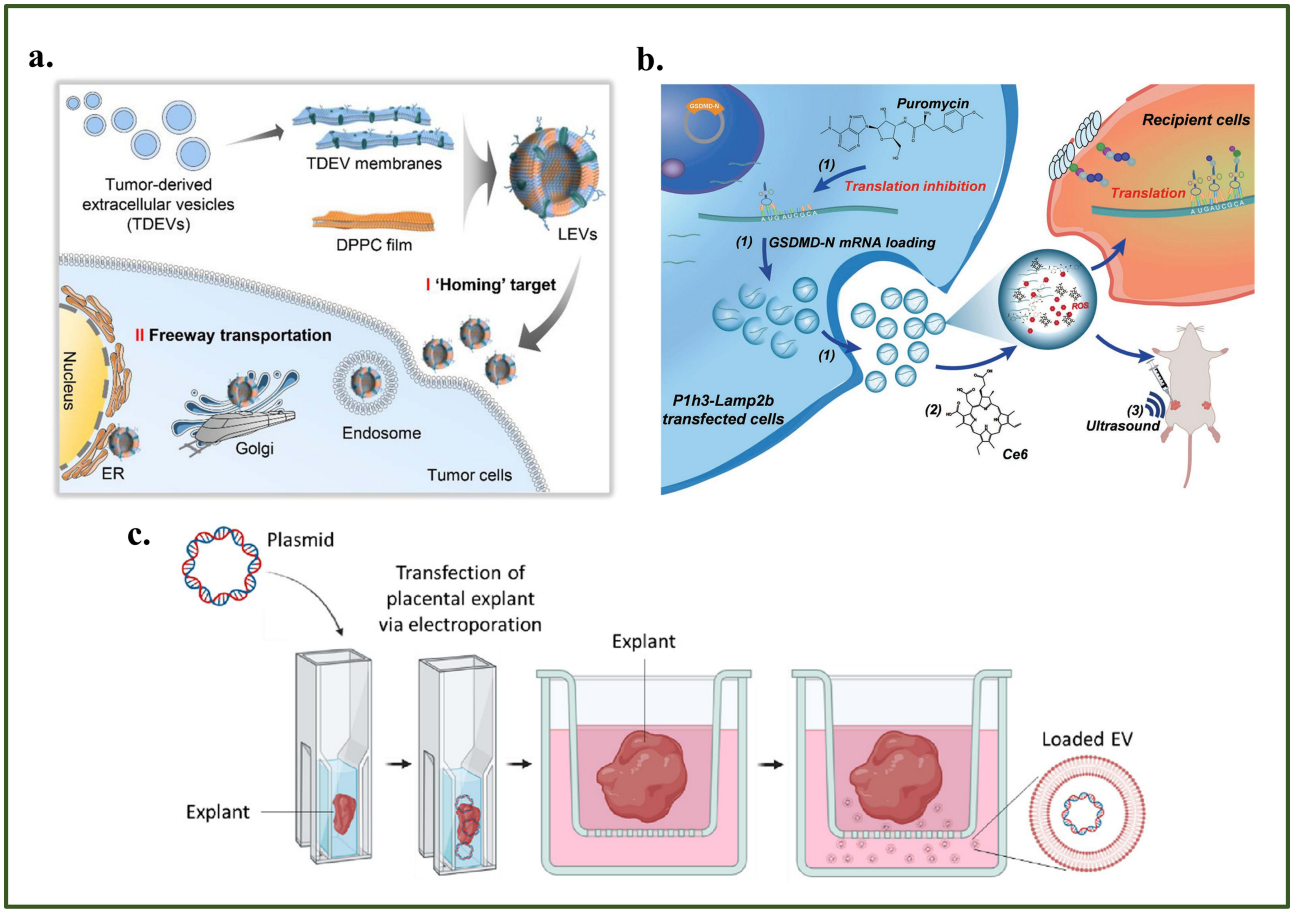

Figure 7 (**a**) Preparation of TDEVs isolated from HCC cells (abandoning their contents) and fused with phospholipids to construct TDEV membrane mixed LEVs (Reprinted with the permission from Zhou X, Miao YQ, Wang Y, et al. Tumour-derived extracellular vesicle membrane hybrid lipid nanovesicles enhance siRNA delivery by tumour-homing and intracellular freeway transportation. *J Extracell Vesicles*. 2022;11(3):e12198;[25] Copyright © 2022 The Authors. Journal of Extracellular Vesicles published by Wiley Periodicals, LLC on behalf of the International Society for Extracellular Vesicles). (**b**) Schematic of the preparation of extracellular vesicle-based GSDMD-N mRNA delivery system (ie EVTx) (Reprinted with the permission from Xing YQ, Zhang FY, Ji PP, et al. Efficient delivery of GSDMD-N mRNA by engineered extracellular vesicles induces pyroptosis for enhanced immunotherapy. *Small*. 2023;19:2204031;[181] Copyright © 2023 Wiley-VCH GmbH). (**c**) Schematic illustration of electroporation-mediated transfection of placental tissue with plasmids, followed by EV collection (Reprinted with the permission from Kang MT, Hisey C, Tsai BD, et al. Placental extracellular vesicles can be loaded with plasmid DNA. Mol Pharmaceutics. 2023;20(4):1898–1913;[186] Copyright © 2023, American Chemical Society).

mRNA

mRNAs are a class of single-stranded RNA transcribed and synthesized from DNA with genetic information. They can control protein synthesis and are an essential genetic material in cells.[179] mRNAs are gradually becoming the mainstay of gene therapy drugs due to their low risk of incorporation into the host genome, rapid protein synthesis, and transient effects of on-demand treatment.[180] In gene therapy, mRNAs are typically used as a source of proteins for the recipient cells. For example, Xing et al constructed a GSDMD-N mRNA delivery system (EVTx) (Figure 7b) based on EVs.[181] GSDMD protein is one of the key molecules for the implementation of pyroptosis. In their work, chlorine e6 (Ce6), a hydrogen sensor, and an HER2 antibody were added to the surface of EVs for the targeted delivery of GSDMD-N mRNA to HER2 (+) breast cancer cells. In normal tumor cells, GSDMD-N mRNA expression was inhibited by the optimized puromycin translation. When the EVs were armed with Ce6, they were further inactivated by sonodynamic therapy, thus allowing GSDMD-N translation and inducing pyroptosis, and then generating the key protein GSDMD that induced cell scorching in the breast tumor mouse model. In addition, our team also utilized the advantages of mRNA therapy to encapsulate extracellular-matrix α1 type-I collagen (COL1A1) mRNA into EVs, and the mRNA-containing EVs induced the formation of collagen-protein grafts, thus effectively reducing the loss of collagen caused by photoaging in mouse models.[32] In another study, using a similar method, the exosome-encapsulated low-density lipoprotein receptor (Ldlr) mRNA was used to treat familial hypercholesterolemia in mice with Ldlr deficiency (Ldlr (-/-) mice). The results

showed that the encapsulated mRNA was stable in the EVs and could be converted into functional proteins in receptor cells, proving that the exosome-mediated Ldlr mRNA delivery could effectively restore the expression of Ldlr. Therefore, this platform could be used to treat the familial hypercholesterolemia in mice.[182] The above results indicate that EVs are superior carriers for the delivery of mRNA because they can prevent the mRNA from sensitive enzymatic degradation in the circulation, thus achieving a high stability after administration.[183]

Delivery of DNAs by EVs

DNA plays an integral role in the transmission of biological information as a source of all proteins produced. Several studies have reported that EVs can stably deliver DNA and enable its stable expression in recipient cells.[184] At present, EVs are widely used as delivery vehicles to deliver therapeutic DNA fragments, including plasmids, DNA aptamers, and ASOs. Herein, we focus on summarizing plasmids and ASOs delivered by EVs owing to the limited number of studies on DNA aptamers.

Plasmids

Plasmids, as one of DNA gene drugs, are widely used in gene therapy because they can replicate transcription to generate the desired gene fragments.[7] Nguyen et al loaded EVs with DNA plasmids encoding GFP by electroporation, and used these DNA-loaded EVs to transfect THP-1-derived macrophages. The results showed that GFP was stably expressed in macrophage subpopulations.[185] Kang et al used placenta-derived EVs to load plasmids, and found that the loading efficiency of plasmid into EVs was determined by the dose and size of exogenous plasmid DNA. Their study showed that the placenta could produce large quantities of EVs for safe drug delivery and also demonstrated the potential of EVs as DNA delivery vehicles (Figure 7c).[186] In addition to the direct delivery of plasmids via EVs, plasmids can also be applied to transfect cells to secrete EVs with target genes. Kanuma et al successfully constructed an OVA-Ag fused to a CD63-expressing plasmid to deliver the Ag into EVs, which increased immunogenicity of the DNA vaccine. They generated OVA-carrying EVs by in vitro transfection using a plasmid DNA encoding OVA-Ag fused to CD63 (pCD63-OVA). Mice vaccinated with pCD63-OVA showed effective Ag-specific T-cell responses.[187] Overall, both direct delivery of plasmids by EVs and indirect delivery by transfection of plasmids into recipient cells, which in turn generate EVs with mRNA, are commonly used for disease treatment, thus providing a rapid way to modulate diseases.

ASOs

ASOs are a class of single-stranded nucleotides that recognize RNA. When ASOs bind to the target RNA sequence, they cause the target gene to be degraded by RNase H, thereby blocking the translation of the disease-causing gene.[188] In addition to this, ASOs can supplement the target gene sequence to form a double-stranded DNA/RNA structure at the target site. It further forms a strong spatial site resistance, thus inhibiting gene function.[189,190] However, owing to the instability of DNA, ASOs have been chemically modified to enhance their suitability. For example, converting a phosphodiester bond to a phosphorothioate bond or introducing a substituent at the 2' position of a sugar in a nucleic acid can solve the stability problem of ASOs.[191,192] However, this strategy has several by some technical problems. The phosphorothioate modification is highly resistant to nucleases and is recognized by RNase H, which results in occupying all positions of ASOs. In contrast, sugar-modified nucleic acids form extremely robust double strands due to their high RNA-binding strength, and ASOs will not be easily recognized by RNase H.[193] Although some problems have been solved, some limitations remain. Moreover, the delivery of ASOs is limited by complex structural modifications. Therefore, a suitable delivery carrier is urgently needed.

Recently, Philip et al compared nucleic acid-modified ASOs (LNA ASOs) encapsulated in EVs with oral LNA ASOs. They found that EVs-LNA ASOs effectively resulted in a reduction of target genes, whereas little change in target gene expression was found with oral LNA ASOs alone.[194] Furthermore, in immune system diseases, EVs-mediated delivery of ASOs has also shown its advantages. ASOs of miR-210 (ASO-210) have been shown to alleviate psoriasis by blocking the development of immune imbalance and psoriasiform inflammation. Zhang et al found that EVs-ASO-210 reduced psoriasis symptoms better than ASO-210 and EVs-IFNγ alone, their schematic representation of the pathogenesis of psoriasis alleviation was shown in Figure 8a.[195]

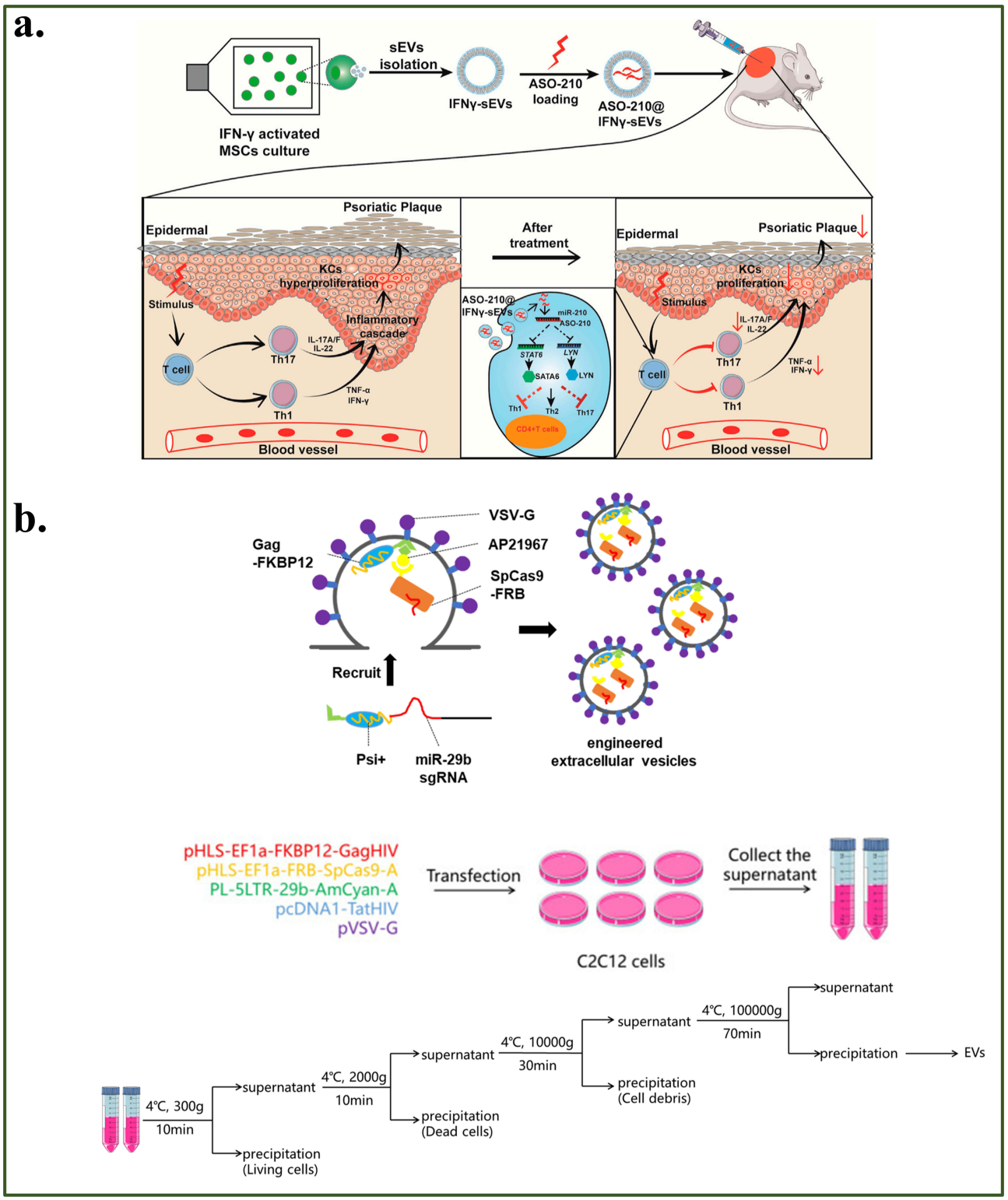

Figure 8 (**a**) Schematic representation of the pathogenesis of psoriasis alleviation by ASO-210@IFNγ-sEVs (Reprinted with the permission fromZhang WX, Lin JX, Shi PL, et al. Small extracellular vesicles derived from MSCs have immunomodulatory effects to enhance delivery of ASO-210 for psoriasis treatment. *Front Cell Dev Biol*. 2022; 2022:10842813;[195] Copyright © 2022,The Author(s)). (**b**) Schematic of tumor-derived exosome-loaded CRISPR/Cas9 targeting PARP-1 in combination with cisplatin (Reprinted with the permission from Chen R, Yuan WL, Zheng YJ, et al. Delivery of engineered extracellular vesicles with miR-29b editing system for muscle atrophy therapy. *J Nanobiotechnol*. 2022;20(1):304;[199] Copyright © 2022, The Author(s)).

Delivery of CRISPR/Cas9 by EVs

The cluster regular interval short palindromic repeat sequence CRISPR/Cas9 system performs gene editing through DNA splicing technology and is widely used in gene therapy; however, it has limited clinical applications due to delivery methods and safety issues.[196] In recent years, EVs have been proposed as a delivery vector for CRISPR/Cas9. Xu et al modified EVs with chimeric antigen receptors as a biosafety delivery platform for the CRISPR/Cas9 system, and the results showed that the CRISPR/Cas9 system could effectively release the anti-MYC oncogene in vivo and in vitro.[197] Other studies have found that cancer cell-derived EVs can selectively aggregate at ovarian cancer tumors sites in SKOV3 heterozygous mice. Therefore, EVs loaded with CRISPR/Cas9 were used to inhibit the expression of poly ADP-ribose polymerase-1 (PARP-1), which induced the apoptosis of ovarian cancer cells.[198] The properties of EVs, combined with the gene editing capability of CRISPR/Cas9, create many novel therapeutic targets and ideas for disease treatment. In a recent study, the inhibition of miR-29b was shown to reduce muscle atrophy, and the CRISPR/Cas9 system was delivered to EVs to construct EVs-Cas9-29b, showing favorable functional effects regarding miR-29b inhibition by gene editing in a specific and rapid manner.[199] A schematic of the engineered extracellular vesicles it prepared is shown in Figure 8b. In summary, EVs provide a safe, effective, and rapid delivery platform for CRISPR/Cas9.[200,201] Although EVs-CRISPR gene editing is rarely used in clinical applications at present, it has proven to be promising and potentially exploitable in preclinical cancer gene suppression studies.[202–205]

Conclusion and Perspective

Recently, gene therapy has been widely used to treat various diseases. Traditional gene therapies, including gene drug therapy and gene editing technology, have shown excellent therapeutic effects. Although some gene drugs such as fomivirsen, mipomersen, defibrotide, and patisiran have been approved by the FDA for clinical use, some limitations (such as easy degradation, low targeting, and high cytotoxicity) still exist. Therefore, many studies focus on developing drug delivery vehicles with high transfection efficiency. Herein, we summarized the advantages and disadvantages of some drug delivery vehicles, as shown in Table 6. In the early years, viral vectors were the most widely used vehicles for the delivery of gene drugs because they possess outstanding transduction efficiency. Recently, many gene therapy products based on viral vectors (such as adenoviral vector, adeno-associated virus, retrovirus, herpes simplex virus, and lentiviral) have been approved for the treatment of cancer, infectious diseases, and single-gene disorders. Adenoviral vector is a class of DNA viruses with a genome size of 34–43 kb. It is one of the

Table 6 The Advantages and Disadvantages of Some Drug Delivery Vehicles for Delivering Gene Drugs

Drug Delivery Vehicles		Advantages	Disadvantages	Ref
Virus vector	Adenoviral vector	High level of gene expression; High transfection efficiency.	High immunogenic	[206]
	Adeno-associated virus	Long-term stable gene expression; Low immunogenic.	High immune side effects	[207]
	Retrovirus	Good ability to evade the immune system; The ability to transport large DNA cargoes and polygenes; Inherent or engineered cell-specific cleavage properties.	High risk of insertional mutagenesis	[208]
	Lentiviral	Long-term stable gene expression; Low risk of insertional mutagenesis.	Limited genetic transport capacity	[209]
Non-viral vectors	Liposome	Good biocompatibility; Low immunogenic; Low cytotoxicity	High lysosomal clearance efficiency	[210]
	Lipid nanoparticles	Good biocompatibility; Low immunogenic; Low cytotoxicity	High lysosomal clearance efficiency	[211]
	Inorganic nanoparticles	Good biocompatibility; High drug-loading efficiency	Complicated preparation	[212]
	Metal nanoparticles	Easy modified	High cytotoxicity	[213]
	EVs	Good biocompatibility; Low immunogenic; Low cytotoxicity; Low lysosomal clearance efficiency	Low yield	[214]

first viral vectors to be used clinically for gene therapy in vivo and presents the advantages of a high level of gene expression and high transfection efficiency. However, its high immunogenicity limits its further application. Although adeno-associated virus with a DNA genome of 4.8 kb is currently the most widely used viral vector for gene therapy in vivo, it exhibits long-term stable gene expression and low immunogenicity. In addition, retrovirus and lentiviral vector are also used to deliver gene drugs. However, they may cause various toxic and immune side effects which greatly limit their clinical applications. Therefore, in order to overcome these drawbacks, non-viral vectors, including liposomes, lipid nanoparticles, and EVs, have been widely applied to deliver gene drugs. Compared with viral vectors, non-viral vectors show a lower transfection efficiency, but possess excellent properties such as good biocompatibility, low immunogenicity and low cytotoxicity. Among them, EVs can inherit the properties of parent cells and obtain some components of parent cells. Moreover, they can be easily engineering-modified and show a higher delivery efficiency than viral vectors. Therefore, in this review, we summarize the biogenesis and composition of EVs, commonly used methods for EVs generation, types of isolation and purification, as well as strategies for engineering EVs and loading of EVs with drugs.

Though very promising, this area is still suffering from some severe limitations during its development from the bench to the bedside. For example, recently, Codiak BioSciences, a leading company in the exosome industry, announced its bankruptcy though some of their clinical trials have been carried out in several stages. One of the major problems in the exosome industrialization is the significant challenge in the scale-up production. Ideally, we need a technique that can scale-up the production of EVs in a short time, and can easily engineer and load gene drugs. Currently, the commonly used methods are difficult to achieve large-scale commercial production of EVs. Chang et al reported a nanosecond pulse electroporation (nsEP) device that can open the cell nuclear membrane without damaging by applying ultra-high frequency electrical stimulation to the cell. Since nsEP has an amplitude width of only 100–1000 ns, it can instantaneously apply a stimulation of more than 1000 V to the cell. This can enhance pDNA transfection and has the potential to stimulate the massive production of EVs.[215] Although the CNP technology developed by our group can increase the yield of EVs and the loading efficiency of pDNA, its commercial production is still under way. Therefore, more works are urgently required to translate the techniques developed in the laboratory into commercialization for the EVs production, engineering and drug loading. In addition, gene therapy can be further achieved by genetically modifying vectors for targeted delivery to cells or tissues. For cell-derived EVs, it not only mediates the delivery of gene therapy molecules or other drug molecules, but also allows the mounting of target cell-specific recognition sequences on the surface of EVs, which are used to achieve targeted delivery of cargo. Moreover, engineered EVs will facilitate the encapsulation of plasmids or vectors because of their high nucleic acid-binding properties, thereby improving the loading efficiency. In conclusion, the advancement and optimization of gene editing technology with the engineering modification of EVs is a knockout punch for EVs-mediated gene drug therapy to enter the clinical era. The continued development of EVs-based mediated drug delivery systems will power targeted gene therapy, making it a therapeutically promising approach for treating diseases with malignant progression. Therefore, we should also devote a precise search to the causative genes and therapeutic targets of various diseases. Through EVs-mediated gene therapy, the expression of disease-causing genes or molecules can be regulated for the development of precision medicine.

Acknowledgments

We acknowledged the support of BioRender. Figures 3, 5 and 6 were created with BioRender.com.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This manuscript is partially supported by the National Natural Science of China (21HAA01203, Z.Y.), Foundation of Jilin Science-Technology Committee (20230402042GH, Z.Y.) and Natural Science Foundation of Zhejiang Province of China (LQ18H020001, X.Z.).

Disclosure

The authors report there are no competing interests in this work.

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