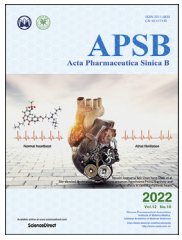
Acta Pharmaceutica Sinica B 2022;12(10):3822–3842

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com



REVIEW

Extracellular vesicles: Emerging tools as therapeutic agent carriers

Shan Liu[a,b,†], Xue Wu[c,†], Sutapa Chandra[b], Christopher Lyon[b], Bo Ning[b], Li jiang[a], Jia Fan[b], Tony Y. Hu[b,*]

[a]*Sichuan Provincial Key Laboratory for Human Disease Gene Study, Department of Medical Genetics, Department of Laboratory Medicine, Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, Chengdu 610072, China*
[b]*Department of Biochemistry and Molecular Biology, Center for Cellular and Molecular Diagnosis, School of Medicine, Tulane University, New Orleans, LA 70112, USA*
[c]*The M.O.E. Key Laboratory of Laboratory Medical Diagnostics, the College of Laboratory Medicine, Chongqing Medical University, Chongqing 400016, China*

Received 25 January 2022; received in revised form 2 April 2022; accepted 28 April 2022

KEY WORDS

Extracellular vesicle;
Therapeutic agent;
Delivery carrier;
Outer membrane vesicle;
Cancer therapy;
Infectious disease vaccine;
Regenerative medicine;
Gene therapy

Abstract Extracellular vesicles (EVs) are secreted by both eukaryotes and prokaryotes, and are present in all biological fluids of vertebrates, where they transfer DNA, RNA, proteins, lipids, and metabolites from donor to recipient cells in cell-to-cell communication. Some EV components can also indicate the type and biological status of their parent cells and serve as diagnostic targets for liquid biopsy. EVs can also natively carry or be modified to contain therapeutic agents (*e.g.*, nucleic acids, proteins, polysaccharides, and small molecules) by physical, chemical, or bioengineering strategies. Due to their excellent biocompatibility and stability, EVs are ideal nanocarriers for bioactive ingredients to induce signal transduction, immunoregulation, or other therapeutic effects, which can be targeted to specific cell types. Herein, we review EV classification, intercellular communication, isolation, and characterization strategies as they apply to EV therapeutics. This review focuses on recent advances in EV applications as therapeutic carriers from *in vitro* research towards *in vivo* animal models and early clinical applications, using representative examples in the fields of cancer chemotherapeutic drug, cancer vaccine, infectious disease vaccines, regenerative medicine and gene therapy. Finally, we discuss current challenges for EV therapeutics and their future development.

*Corresponding author. Tel.: +1 504 988 5310; fax: +1 504 988 1611.
E-mail address: tonyhu@tulane.edu (Tony Y. Hu).
[†]These authors made equal contributions to this work.
Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2022.05.002

1. Introduction

Extracellular vesicles (EVs) are natural nanoscale phospholipid bilayer structures that are actively released by eukaryotic prokaryotic cells[1,2]. At their discovery in the late 1980s, EVs were considered to be cellular "junk"[3], but research and methodological developments have since led researchers to realize that EVs play critical roles in cell-to-cell communications that regulate both homeostatic and disease processes through their actions on immune function, tissue repair, and cell growth. Recent advances have significantly increased understanding of EV interactions and effects upon their recipient cells.

EVs are detectable in all vertebrate body fluids and contain DNA, RNA, proteins, lipids, and metabolites from their parental cells, some of which are specific for their parental cell type or its physiologic or disease status[4]. Analysis of these cargoes can therefore provide evidence for early disease diagnosis and real-time evaluation of disease severity to allow prognostic evaluation and treatment monitoring. EVs also can also function as excellent nanocarriers for drug delivery. Synthetic nanocarriers fabricated from cationic polymers, cyclodextrin, lipids, etc. have been examined for delivery of therapeutic agents[5], but their clinical application can be hindered by their toxicity, immunogenicity, low loading efficiency, and preferential accumulation in the liver and spleen[6]. EVs, by contrast, have strong potential in applications ranging from cancer therapy to regenerative medicine due to their high biocompatibility and low toxicity and immunogenicity. EVs can also evade the mononuclear phagocytic system to show relatively stable in the circulation and penetrate multiple biological barriers to improve their accumulation at targeted sites[7]. EVs can also be modified by bioengineering approaches to achieve targeted delivery of their therapeutic cargoes to specific sites[8].

In this review, we summarize recent developments, advances, and representative examples that apply to the development of EV therapeutic biomedical applications.

2. Classification

EVs are generally classified into three major subpopulations by their diameters and biogenesis mechanisms: exosomes (30−120 nm), microvesicles (50−1000 nm), and apoptotic bodies (50−2000 nm)[9] (Fig. 1). Exosomes are generated by inward budding of the endosome membrane to form multivesicular bodies (MVBs) that fuse with the plasma membrane to release mature exosomes *via* a closely regulated process. Conversely, microvesicles and apoptotic bodies are generated by outward budding of the plasma membrane by different processes in viable cells and in cells undergoing proGrammed death, respectively[10]. However, all three EV populations can overlap in size, surface markers, and composition to prevent reliable isolation of pure EV samples of specific subtypes by methods in current use. Given this lack of precision, we use the generic term "EV" rather than exosome or microvesicle in this review when describing results of studies that report the innate or engineered properties of these vesicles.

This does not imply that these EVs carry the same markers and mediate similar functions. One study that analyzed the proteomes of large and small EVs (100−800 nm *vs.* 30−150 nm) identified multiple proteins that were preferentially enriched in large EVs (ATP5F1A/B, DHX9, GOT2, HSPA5, HSPD1, MDH2, STOML2) and small EVs (CD9, CD44, CD63, CD81, CD82, PDCD6IP, SDCBP, TSG101)[11]. However, this study did not attempt to determine if the EVs derived from the cytoplasmic or endosomal membrane (ectosomes *vs.* exosomes), although gene ontology analysis indicated the endosome associated proteins were over-represented in the small EV fraction, as would be expected due to the size range of exosomes. Another study that attempted to identify markers differentially expressed in the proteomes of exosomes and endosomes of HeLa cells reported that EVs that express CD9 and CD81 but little CD63 primarily derive from the plasma membrane, while those that express CD63 but little CD9 predominantly derive from the endosomal membrane[12]. However, this has yet to be replicated in other cell types and under different conditions. EV subtype classification and analysis is also further complicated by potential differential contributions from distinct exosome subtypes, since one group has reported that exosomes can be further divided into three subtypes ([exomeres ∼35 nm], and small [60−80 nm] and large [90−120 nm] exosomes) that differ in their physical properties (diameter, zeta potential, and stiffness) and cargo compositions[13].

Despite the lack of precision in EV subtype isolation, several studies have reported that EVs from different sources carry specific bioactive components from their parental cells that can confer specific functional properties. EVs are thus often frequently classified based on their cell or tissue source and associated regulatory activity or application. EVs derived from immune cells represent one example. DC-derived EVs are of great interest for vaccine applications since they carry peptide: MHC complexes and their co-stimulatory molecules, and integrins and other proteins involved in regulating adaptive and innate immune responses[14]. EVs secreted by NK cells carry NK marker and cytotoxic molecules (*e.g.*, CD16, CD56, granzyme and perforin)[15] that can promote NK cell proliferation and induce cytotoxic responses[16]. Macrophage-derived EVs carry factors that can regulate pro-inflammatory responses and induce macrophage polarization. By contrast, MSC-derived EVs, which can be produced at large scale[17], can promote tissue repair and maintain tissue homeostasis by delivering trophic factors, signal molecules, regulatory RNAs, proteins, and other factors[18]. That can exert therapeutic effects on their recipient cells. EVs secreted by diseased cells can also carry factors that permit them to serve as the basis of vaccine strategies. This included tumor derived EVs, that can serve as candidates for cell-free cancer vaccines. However, safety is a priority when using EVs derived from infected or malignant cells, since these EVs can transfer material that can initiate an infection or play important roles in tumor progression and immune responses[19]. Bacteria also secrete vesicles similar to vertebrate EVs, and EVs secreted by bioengineered bacteria can function as excellent vaccine carriers since they usually display multiple pathogen-associated molecular patterns that can function

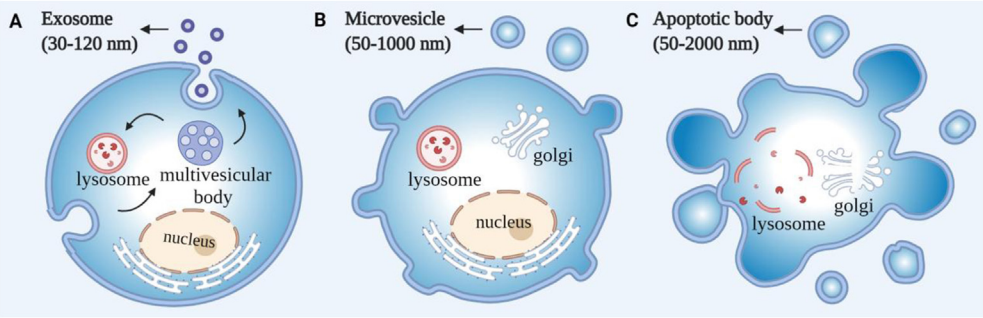


Figure 1 Biogenesis and classification of extracellular vesicle subtypes. (A) Exosomes are released by the fusion of multivesicular bodies with the plasma membrane. (B) Microvesicles are generated by outward budding of the plasma membrane. (C) Apoptotic bodies are produced by the budding of the cell undergoing programmed death.

as adjuvants to stimulate a robust immune response to the pathogenic-specific factors they carry.

3. EV roles in pathological and physiological conditions

3.1. Mechanisms of EVs-mediated intercellular communication

The targeted delivery of EV cargoes to recipient cells to play essential roles in intercellular signal transduction and tissue homeostasis. EV-mediated cell-to-cell communication is mediated by three primary mechanism: receptor−ligand interactions, direct membrane fusion and endocytosis[20] (Fig. 2), which can respectively induce signaling cascades, transfer regulatory factors into the plasma membrane and cytosol, or release such factors into the cytosol after EV uptake by endosomes.

EVs can mediate immunomodulatory effects without endocytosis since EV ligands can initiate downstream signaling cascades *via* interactions with surface receptors on target cells. For example, major histocompatibility complex (MHC) present on EVs derived from dendritic cells (DCs) can directly interact with the T cell receptor to affect immune responses[21]. Similarly, EVs released by metastatic melanoma cells that express high levels of the proGrammed cell death ligand-1 (PD-L1) can suppress the function of $CD8^+$ T cells and facilitate cancer growth by interacting with proGrammed cell death protein-1 (PD-1) expressed on these cells[22].

Multiple studies have, however, shown that EVs can fuse with recipient cells to transfer their membrane and vesicular cargoes. An early study that incubated recipient cells with EVs that were membrane-labeled with a fluorescent lipid dye found that the EV label was transferred to the recipient cells *via* membrane fusion[23]. Subsequent studies found that EV fusion events released EV cargoes into the cytosol that could have regulatory effects, as exemplified by one early study that determined that EV miRNA cargo transfer could repress mRNA translation in recipient cells[24].

EVs can transfer their cargoes to exert regulatory effects *via* a receptor-independent fusion with the plasma membrane[25]. However, mounting evidence indicates that EV endocytosis is the primary means of EV-mediated cell-to-cell communication and can occur *via* five different mechanisms: clathrin-, caveolin-, and lipid-raft-mediated endocytosis, macropinocytosis, and phagocytosis[26]. Most EV recipient cells employ clathrin-mediated endocytosis for EV uptake, in a process that involves clathrin assembly around membrane-bound EVs, followed by membrane bending and invagination to form clathrin-coated vesicles, that bud inward and undergo scission from the plasma membrane to enter the cytoplasm, and fuse with early endosomes after release of their clathrin shells[27]. However, there is evidence that EV uptake can also occur through other mechanisms. For example, caveolin-dependent endocytosis can also produce small plasma membrane invaginations that produce intracellular vesicles[28]. Caveolin-1, the primary protein component of these vesicles, is also reported to regulate EV endocytosis and internalization[29], but there is conflicting data on the relevance of caveolin-dependent endocytosis for EV uptake. One group has reported that caveolin-1 can promote EV uptake in epithelial cells[30], while another has indicated that caveolin-1 inhibits EV uptake in glioblastoma cells and fibroblasts *via* a signal transduction-mediated process[31]. Lipid raft-associated membrane invagination events may also permit EV uptake and subsequent fusion with early endosomes[32]. Macropinocytosis, a process in which the plasma membrane deforms to envelop extracellular fluid, has also been shown to regulate EV entry into the cytosol through a mechanism that requires cholesterol, Na^+/H^+ exchange, and phosphatidylinositol-3-kinase (PI3K) activity[33]. Finally, EV uptake can also occur *via* phagocytosis, which primarily occurs in immune cells such as macrophages and DCs, through a receptor-mediated actin polymerization process that induces membrane invagination to engulf the contacted material and target it to the phagosome pathway[34]. Several of these mechanisms have also been observed to regulate EV entry into the same cells, to complicate interpretation of this process[35].

EVs which have fused with early endosomes after entering recipient cells, can accumulate in the endosomal compartment to form multivesicular bodies and most of them may be targeted to lysosomes/autophagosomes, leading to EV degradation, which could provide metabolites to the recipient cells; fuse with plasma membrane to induce extracellular EV release for recycling, or these EVs may fuse with the endosomal membrane to release their contents into the cytosol to exert effector functions[36] (Fig. 2). This final process is still poorly understood but required for regulatory EV cargo transfers that participate in various pathological or physiological responses in recipient cells[37]. However, more is known about factors that influence the efficiency of EV internalization. Surface factors present on EVs and their potential recipient cells can regulate EV uptake, and conditions that enhance the cleavage of these factors, including temperature, can inhibit EV

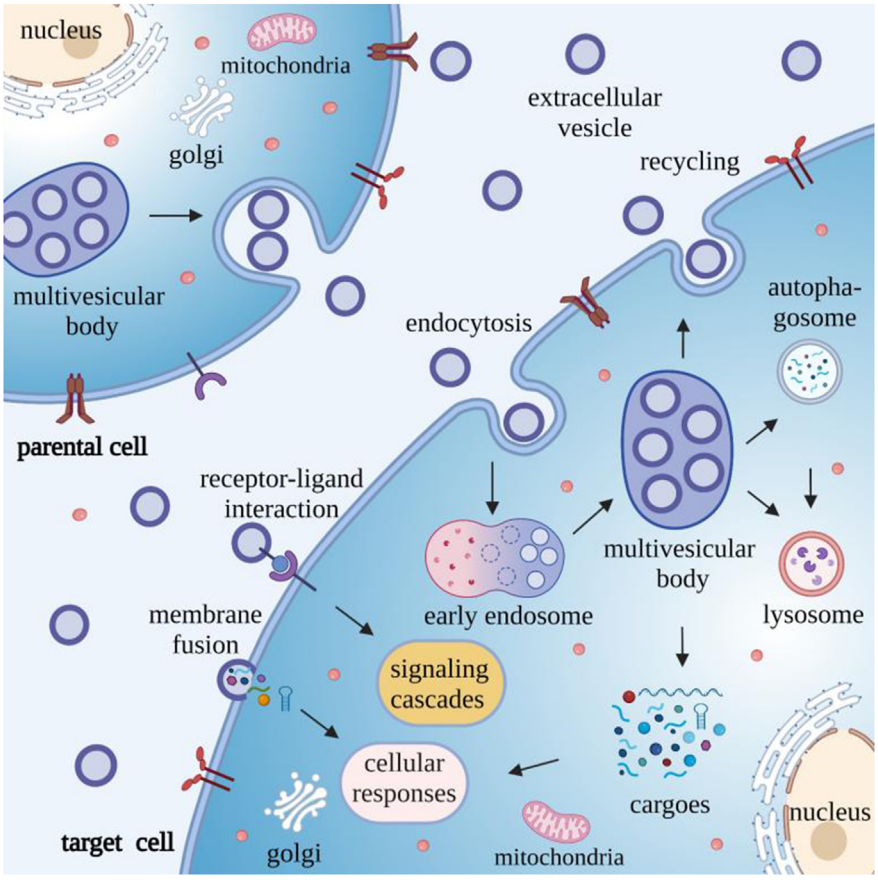


Figure 2 EVs-mediated intercellular communication. EV interact with target cells by three mechanisms to facilitate intracellular communication: 1) direct membrane fusion, which release their contents into the cytoplasm of the recipient cell to where they can exert regulatory effects; 2) receptor−ligand interactions, which can induce signaling cascades; and 3) endocytosis, where EVs first accumulate in endosomes to form MVBs, which then fuse with lysosomes/autophagosomes for degradation, fuse with the plasma membrane for recycling, or release their contents into the cytosol to allow the captured EV cargoes to exert regulatory functions.

uptake[20]. Microenvironmental pH has also been reported to influence EV release and uptake, partially by altering the lipid composition of the EV membrane to enhance their fusion efficiency with the membranes of recipient cells[23].

3.2. *EV roles in physiological conditions*

EVs are ubiquitous and can transfer membrane proteins, signaling molecules, nucleic acids, and other materials from their parental cells to regulate multiple physiologic processes. For example, EVs secreted by antigen-presenting cells (APCs) carry MHC proteins and signaling molecules that can present antigenic peptides to T cells to induce effector activity[38], although this immune response induced is not as effective as that induced by APCs and the reason for this difference is not clear[39]. EVs can also carry bacterial components from macrophages infected with an intracellular pathogen (*e.g.*, *Mycobacterium avium*) to uninfected macrophages to activate them *via* a toll-like receptor ligand dependent mechanism[40]. In addition to these immune effects, EVs also regulate intercellular communication associated with other critical processes, including angiogenesis, cell proliferation and apoptosis, tissue homeostasis and remodeling, and reproduction and development[41,42]. For example, EVs secreted by platelets, which regulate the clotting response following tissue injury, contain α-granules, coagulation factors, growth factors, and RNA species that play important roles in wound healing through their actions to modulate coagulation, inflammation, cell growth, and stem cell proliferation, migration, and differentiation[43]. Similarly, EVs secreted by Schwann cells carry miRNAs that promote cell-to-cell communication and enhance peripheral nerve regeneration after nerve damage[44].

3.3. *EV roles in pathological conditions*

EVs secreted by injured or diseased cells and tissues have also been implicated in initiating or promoting pathological responses associated with malignant, chronic, and infectious disease states. Tumor-derived EVs derived from different cell types in the tumor microenvironment (TME), including cancer, stromal, and immune cells, carry genetic material and regulatory factors that participate in various pathological changes, including TME remodeling, the immune response to cancer antigens, therapy resistance, and tumor invasion, metastasis, and migration[45,46]. These EVs can express FasL, NKG2D, TGF-β, and PD-L1 to induce T cells apoptosis and suppress NK cells cytotoxicity to facilitate tumor escape from immune surveillance[47]. The TME is a highly complex and dynamic system where metabolic remodeling may provide the energy or additional materials required for tumor survival, growth,

and migration, and non-coding RNAs present in tumor-derived EVs may influence these mechanisms[48]. TME stromal cell EVs can also influence tumor pathology by transferring their cargoes to neighboring cells to triggering intracellular signaling events that inhibit apoptosis and promote tumor proliferation[49]. Conversely, EVs secreted by immune cells can inhibit tumor development by depleting mesenchymal tumor stromal cells *via* altering signaling pathways that elicit mesenchymal-to-epithelial transition to promote TME development[50].

EVs also play important roles in tissue injury in neurodegenerative disorders, including the transport of specific proteins that accumulate and form protein aggregates that are common pathological characteristics of such disorders and can contribute to disease pathology[51]. For example, EVs secreted by central nervous system tissue of patients with Parkinson's disease contain elevated levels of α-synuclein, the primary component of characteristic protein aggregates associated with this neurodegenerative condition[52]. EV α-synuclein levels also correlate with disease severity and may enhance neuronal injury, since normal neuronal cells exhibit greater apoptosis when exposed to EV containing α-synuclein oligomers *versus* free α-synuclein oligomers, which may promote the spread of neurological injury[53].

EVs have also been reported to mediate the pathology of several other chronic conditions, including cardiovascular, ocular, and endocrine diseases[38,54,55], as well as infectious diseases. For example, EVs released from damaged endothelial cells (ECs) carry multiple bioactive molecules that can increase apoptosis, clot and atherosclerotic plaque formation at recipient ECs[56], while EVs secreted by infected cells can transfer pathogen-derived factors to promote systemic infection or influence the host immune response[57]. Notably, overlaps among host pathways involved in viral packaging and exosome biogenesis have been proposed to permit EVs secreted by cells infected with hepatitis C virus (HCV) or HIV-1 to package and transfer these viral genomes to recipient cells to induce productive infections[58]. Serum EVs from patients with HCV infections carry replication competent viral RNA complexed with factors that can promote HCV replication or stabilize its replication complex (Ago2, miR122, and HSP90), and can transfer the HCV RNA genome to recipient cells to promote infection[59]. These carrier EVs may express few if any viral proteins, unlike HCV virions, and thus avoid a systemic antibody-mediated virus neutralization response[60]. HIV can also employ EV transfer to evade an antibody neutralization response. EV-mediated transfer of the HIV virulence factor Nef can induce a pre-activation state in recipient $CD4^+$T to increase their susceptibility to HIV infection and apoptosis[61]. Nef expression can also activate PI3K signaling to alter endosomal vesicular formation and trafficking to downregulate MHCI expression and cytotoxic immune responses against HIV-infected $CD4^+$T cells[62].

4. EV isolation and quality control requirement for EV therapeutics

EVs, lipoprotein complexes, protein aggregates, and other materials present in biological samples exhibit similar physical properties that complicate the isolation of high purity EVs required for clinical EV applications. Segregation of specific EV subpopulations can be even more difficult since these groups exhibit substantial size overlap and morphological similarity, and lack distinctive markers to permit their specific differential capture[63]. Nevertheless, accurate EV characterization can be important for EV applications, since different EV subtypes may express different factors and thus therapeutic effects may depend upon the reproducible isolation of specific EV subtypes that contain the desired regulatory factors.

4.1. *Isolation strategies*

Several approaches have been used to isolate EVs from biological samples, and each requires trade-offs between purity, yield, and integrity of isolated EV fractions (Table 1).

Ultracentrifugation (UC) remains the gold standard[64,65], and most popular EV, isolation technique[66], and employs differential centrifugation to remove cells, debris, organelles, and large particles from EV source materials before applying high-speed centrifugation to precipitate EVs. UC is low throughput and time-consuming, however, and the EV fractions it produces exhibit highly variable purity, since EVs can co-precipitate with several factors not removed during sample clarification, including protein, DNA and RNA aggregates, lipoproteins, and others materials[67]. Ultrafiltration (UF) approaches that employ size-exclusion membranes to remove large bioparticles from biological samples can be faster and produce higher purity EV fractions than UC[68], although trapping of large sample components may clog membrane pores and reduce EV yields and strong shear forces encountered during filtration can rupture EVs or damage their structural integrity[69]. Size exclusion chromatography (SEC) can also be used to separate EVs from particles that differ in size[70]. In this approach, EV containing biological specimens are fractioned over columns packed with porous beads that have an exclusion diameter less than that of the targeted EV population, and EVs are rapidly eluted while smaller materials are retained on the column[71]. SEC can preserve the structure and maintain the function of EVs, although sample components similar in size to EVs are also isolated in the EV SEC fraction[72]. EV precipitation methods primarily employ polyethylene glycol (PEG) to differentially precipitate larger, less soluble sample components like EVs[73]. PEG-based precipitation is simple, inexpensive, and gentle[74] as it requires only a low-speed centrifugation step to produce concentrated and high-yield EV samples[74]. However, these EV fractions have low purity since EVs co-precipitate with multiple non-specific factors, including large particulates and molecular aggregates[75]. Immunoaffinity precipitation methods using receptors or antibodies that recognize EVs-specific factors are an effective means of obtaining high purity EV isolates[63,76], but EV yields are usually much lower than obtained with PEG-based precipitation methods, other factors can still be pulled down by interaction with the precipitation matrix, and this approach is much more expensive than PEG precipitation[77]. Several EV isolation approaches have also recently been proposed that use different characteristics for EV segregation than standard EV isolation methods. These include microfluidic approaches that can achieve microscale isolation of EVs based on their physical and biochemical properties[78]; asymmetric flow field-flow fractionation (AF4) and nano-flow cytometry (nano-FCM)[65,79]. However, it is unclear if these approaches can be employed for large-scale isolations required to formulate future EV therapeutics, and both they and the current EV isolation studies will require extensive validation studies before they can be employed to isolate EVs for this purpose.

Table 1 Principles and characteristics of isolation methods of EVs.

Type	Method	Principle	Advantage	Disadvantage
By density	Ultracentrifugation	Different centrifugal processes to isolate EVs based on density and mass	Easy to operate; Low cost	Low purity; Low throughput; Time-consuming
By size	Ultrafiltration	Using the filter membrane to remove large bioparticles	High purity; Time-saving	Clogging the nanopores; Damaging the structure and dissoluting EVs;
	Size exclusion chromatography	Large particles such as EVs are unable to pass through column thus rapidly eluting	High purity; Preserving the structure	The contaminations co-eluting with EVs
By solubility	Polymer precipitation	Changing the solubility of the solution	Easy to operate; Low-cost; Getting concentrated and high-yield EVs	Poor specificity; The contaminations co-precipitating with EVs
By immunoaffinity	Immunoaffinity magnetic beads; Immunoaffinity chromatography; Plate-mounted immunoaffinity	The surface markers of EVs interacting with antibody	High specificity; High purity	High-cost; Low throughput; Relying on reliable markers
Emerging methods	Microfluidics	Using various methods achieve microscale isolation based on their physical and biochemical properties	High purity; Low sample consumption; Low cost	Lack of standardization; Clogging the probe
	Asymmetric flow field-flow fractionation	Based on the particles density and hydrodynamic properties	Label-free; Gentle; Getting EVs subpopulations	Complex processes; Low throughput
	Nano-flow cytometry (nano-FCM)	Based on the particles of polydispersity, charge characteristics and surface markers	High throughput; High resolution	High cost; Professional personnel

4.2. *Characterization strategies*

EVs must be characterized after isolation or before and after loading therapeutic agents to ensure that they meet the requirements of their intended application. Various strategies are available to characterize EV size and morphology, and protein, nucleic acid, and lipid composition (Table 2).

EV imaging methods used for high-resolution analysis of EV size and morphological properties include transmission electron microscopy (TEM), cryo-electron microscopy (Cryo-EM), scanning electron microscopy (SEM), and atomic force microscopy (AFM). Dynamic light scattering (DLS) is used to measure the Brownian motion of suspended particles isolated EV samples to estimate the polydispersity index (PDI) of their particle diameters[80]. Nanoparticle tracking analysis (NTA), a DLS-based method, is one of the most common techniques used to calculate EV size distributions and concentrations[81]. Imaging flow cytometry (IFCM) can more accurately assess EV concentration than NTA, since IFCM allows for single-particle quantification based on EV' immunophenotype marker and size rather than bulk quantification[82]. However, EV sizes and concentrations are not frequently estimated by IFCM or several other alternate analysis methods, including nano-FCM, confocal laser scanning microscopy (CLSM), tunable resistance pulse sensing (TRPS) and transmission surface plasmon resonance (TSPR) and frequency-locked optical whispering evanescent resonance (FLOWER).

EVs can also be characterized by analysis of biomarkers positively and negatively associated with EVs, with the International Society for Extracellular Vesicles (ISEV) recommending at least one protein belonging to three distinct categories be analyzed to evaluate the nature and relative purity of EV factions[83]. This includes the analysis of a transmembrane or GPI-anchored proteins that denote the existence of a lipid bilayer, a cytosolic protein that indicate the detected vesicles contain a lumen, and a commonly co-isolated negative control protein that serves as an indicator of sample contamination. Specific factors of interest must also be characterized to ensure that the EV fraction meets the specific need of its research or clinical application (*e.g.*, expresses a surface factor that will be employed for targeted delivery of a therapeutic agent). EV protein composition has been analyzed by standard quantitative techniques, including enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot and mass spectrometry (MS) analyses[84]. EV characterization

Table 2 Principles and characteristics of characterization methods of EVs.

Type	Method	Principle	Advantage	Disadvantage
By physical property (size and morphology)	TEM; Cryo-EM; SEM	Electron radiation	High resolution	High cost; Low throughout; Complex sample processing; Not quantitative
	AFM	Measuring the force between the probe and sample	High resolution	High cost; Low throughput; Not quantitative
	DLS	Measuring the scatter light from EVs in Brownian motion	Easy to operate; Low cost	Not quantitative; Not suitable for polydisperse sample
	NTA	Capturing the Brownian motion of individual particle	Quantitative; Suitable for monodisperse and polydisperse samples	Affecting by the instrument parameter settings
	Imaging FCM	Based on FCM and fluorescence imaging	Sensitive; High throughput; Low sample volume	High cost; Professional personnel
	Nano-FCM	FCM based on nanopore	Quantitative; Low sample volume	High cost; Professional personnel
	CLSM	Microscopy imaging after fluorescent label	High resolution; Dynamic visualization	Not quantitative; High cost
	TRPS	Based on the changes of resistance pulses of a single particle through a pore	Quantitative; Low sample volume	Clogging the pore by large particle
	TSPR	Based on free electrons collectively oscillate under the Incident light field	Quantitative; Low sample volume	Noise interference by containments
By compositional property (protein)	ELISA	Immunoaffinity	High throughput; Fast	High cost; Low specific
	SDS-PAGE	Characteristic absorption in the visible spectrum	Easy to operate; Fast	Not quantitative; Low detection limit
	WB	Immunoaffinity	Quantitative; Specific	High cost; Time-consuming
	MS	q/e analysis of small fragments	High specific; Quantitative	High cost; Professional personnel
By compositional property (nucleic acid)	UV−Vis	The characteristic absorption peaks	Low cost; Easy to operate; Fast	Low specific
	qPCR	Amplification of specific genes	High throughput; Low sample volume	Only suitable for known genes
	microarray	Based on the principle of base pairing	High throughput; Specific	High cost; Professional personnel
	NGS	Fluorescence sequencing after RNA reverse transcription	Sensitive; Specific	Low throughput; High cost
By compositional property (lipid)	GC−MS, LC−MS	q/e analysis of small fragments	High specific; Quantitative	High cost; Professional personnel

studies have also employed UV−Vis spectrometry and capillary electrophoresis to quantify nucleic acid levels and size distributions, quantitative real-time polymerase chain reaction (qPCR) to quantify individual nucleic acid targets, and microarray analyses and next-generation sequencing (NGS), which can sensitively detect low abundance transcripts, to survey the expression of a broad array of genes. Less research has been performed to characterize EV lipids, but gas chromatography (GC)−MS and liquid chromatography (LC)−MS studies have been employed for EV lipidomic studies[85].

Reliable EV isolation and characterization protocols are critical for consistent isolation of EVs that have innate therapeutic properties or that have been modified by bioengineering or drug loading to function as therapeutic agents. Several issues still need to be addressed to refine these processes. EV preparation procedures should be streamlined to reduce isolation times and enhance the integrity, stability, and functionality of EV preparations. Most EV characterization methods are expensive, complex, and time-consuming. Improved procedures are therefore needed to achieve high-purity isolations of specific EVs or EV subtypes. Some therapeutic applications may also require selective purification of distinct EV subtypes, which can be challenging due to potential overlaps in their physical properties and surface factors. New EV characterization methods should thus rapidly and accurately distinguish target EV subpopulations by distinctive differences in their physicochemical or biological properties to generate EV preparations with reproducible cell targeting and drug loading properties to produce consistent therapeutic effects. Such new characterization methods are also needed to facilitate the development of improved EV separation methods. Substantial effort is now focused on developing new methods to meet these needs.

5. EVs as therapeutic agent carriers

Some drugs and biomolecules with promising therapeutic effects have low aqueous solubility, are subject degradation, have toxic side effects, and/or lack specificity for the targeted cells or tissues, resulting in poor *in vivo* bioavailability and therapeutic effects. Researchers have thus developed synthetic delivery vehicles based on cyclodextrin, cationic polymers, polymeric nanoparticles, or

liposomes or modified viruses to limit the undesired properties of therapeutic molecules[86,87]. However, these carriers can exhibit relatively high immunogenicity, short circulation times, and preferential accumulation in highly vascularized tissues, including the spleen and liver, rather than disease sites[6,88] partially due to phagocytic cell uptake. Modified viruses, which are mainly used as nucleic acids delivery vectors, can improve uptake rates and may be permit some degree of target specificity, but their inherent immunogenicity renders them highly susceptible to host immune responses that can decrease their efficacy and increase their safety risks[89].

By contrast, EVs have several advantages. Most EVs used in therapeutic applications are derived from human cell lines or primary cultures and thus have low immunogenicity and high biocompatibility since they do not display exogenous factors targeted by the immune system, including the mononuclear phagocytic system, and tend to have greater stability in the circulation due to their important roles in endocrine signaling events[90]. EVs are non-replicating and thus have greater safety profiles than viruses, but like viruses can natively carry surface factors that allow tissue- or cell-selective uptake to increase the effective dose in a target tissue while reducing systemic side effects[91]. EVs can also efficiently transit biological barriers, including blood vessels and the blood−brain barrier (BBB)—a critical feature for drug delivery vehicles[92]—to permit targeted or untargeted delivery of their therapeutic factors and thereby increase bioavailability across such barriers to reduce doses required for therapeutic effects and systemic side effects. In the following sections, we review how these EV properties have been employed in EV applications for cancer therapy, vaccine, regenerative medicine and gene delivery applications.

5.1. *Cancer chemotherapeutic drug carriers*

EVs serve as excellent carriers for both polar and non-polar chemotherapeutic drugs, since they can transport hydrophobic drugs in their lipid bilayer and hydrophilic drugs in their lumen (Fig. 3A). EVs also contain transmembrane and membrane-anchored proteins that can promote endocytosis to improve the efficiency of intracellular delivery of their chemotherapeutic drug[93]. Further, surface factors present on EVs derived from specific cells, including cells bioengineered to express factors that confer specific cell trophisms, can be used to enhance delivery of EV-loaded chemotherapeutics to specific cells or tissues. This can increase the selective drug concentrations and bioavailability in target tissues while reducing cytotoxic side effects caused by drug actions at other systemic locations[94]. Several groups have now used EVs to deliver a broad array of chemotherapeutics, including paclitaxel (PTX), 5-fluorouracil (5-FU), doxorubicin (Dox), celastrol (CEL), β-elemene, curcumin (Cur), and sorafenib (SRF)[95,96], to take advantage of their beneficial drug delivery characteristics.

For example, in an attempt to improve bioavailability during the administration of chemotherapy drugs, one study loaded EVs isolated from cow milk with PTX, a first-line broad-spectrum chemotherapeutic drug that exhibits poor aqueous solubility, substantial toxicity, and rapid degradation[97]. In this study, PTX was directly loaded into the lipid bilayer of these EVs by directly mixing the EV isolates with the PTX solution at a 10:1 volume ratio, after which EVs were precipitated and PBS washed to remove unbound drug, sterile filtered and stored at −80 °C until oral administration[98]. Notably, these EVs showed excellent stability in a simulated gastrointestinal environment, and mice

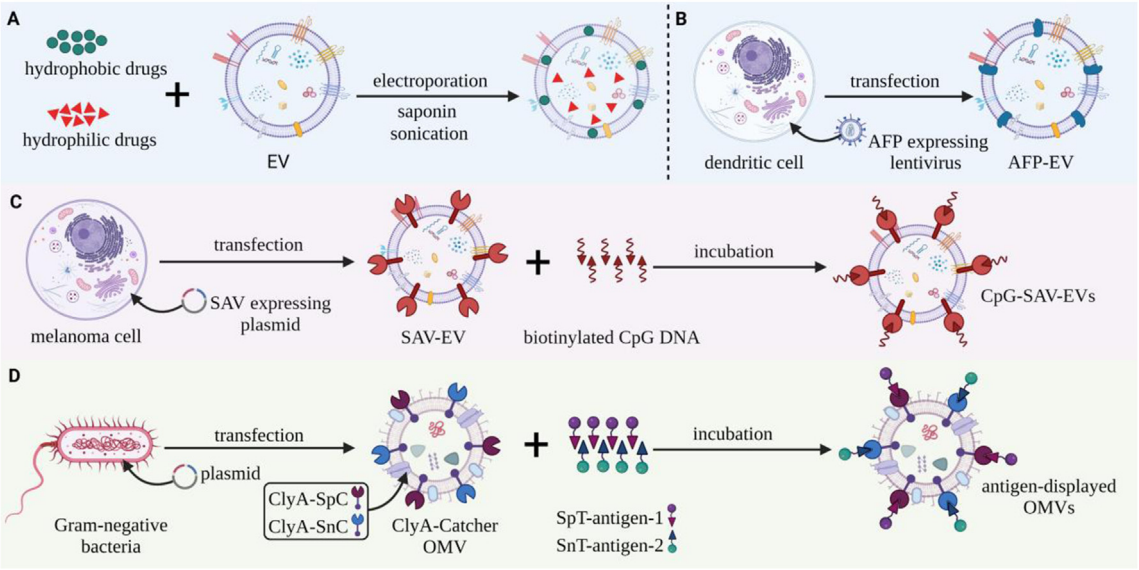


Figure 3 Strategies for loading EVs with different cargoes for cancer therapy. (A) Hydrophobic drugs (green circles) can be loaded into EV lipid bilayers by direct incubation, while hydrophilic drugs (red triangles) can loaded into the EV lumen by EVs by electroporation, sonication, and saponin-mediated membrane permeation. (B) Dendritic cells engineered to express proteins with specific cell tropism (*e.g.*, α-fetoprotein; AFP) by transfection with lentivirus expression vectors can produce EVs useful for cell- or tissue-selective EV targeting. (C) Melanoma cells transfected with streptavidin (SAV) expression vectors produce SAV-modified EVs that can be incubated with biotinylated CpG DNA to produce a CpG-SAV-EV adjuvant. (D) Gram-negative bacteria transfected by plasmids that express two ClyA-"catcher" fusion proteins (ClyA-SpC (red) and ClyA-SnC (blue)) can be used to produce OMVs expressing these catcher activities on their outer membrane. Incubation of these OMVs with proteins modified with the corresponding tags (SpT and SnT, red and blue triangles) permits the formation of an isopeptide bond between them to allow the stable display of these proteins on the resulting EVs.

treated with oral doses of these EVs exhibited greater inhibition of tumor growth and lower systemic and immunogenic toxicity than mice treated with free PTX by intravenous injection[98].

The properties of native EVs have also been employed to promote target EV delivery across other biological barriers than gastrointestinal lining, using synthetic vesicles designed to mimic EVs. This includes the BBB, which prevents >98% of small molecule drugs from entering tumor cells within brain tissue[92], since EVs display better delivery effects than free drugs and liposome-mediated drug delivery. In one study, EVs and synthetic EV analogs (bioinspired nanovesicles: BNVs) loaded with DOX were shown to cross the BBB to demonstrate excellent tumor suppression effects in mice and zebrafish models of glioblastoma. BNVs used in this study were generated by serial extrusion of bEnd.3 brain-derived endothelial cells to produce 500-fold more BNVs than EVs that could be isolated from the same number of cultured cells. Interestingly, both exhibited similar sonication-induced drug-loading capacities and pharmacokinetic parameters, with both exhibiting greater DOX bioavailability with a longer half-life and reduced systemic clearance than free DOX. Treatment of a mouse glioblastoma model with either DOX-loaded EVs or BNVs also significantly decreased tumor volume *versus* mice treated with free DOX or liposome-encapsulated DOX, without inducing weight loss or other sides effects associated with the latter treatments. Thus, EVs and BNVs derived from brain endothelial cells demonstrate promise for targeted drug delivery across the BBB, and EVs and BNVs derived from other cell types may also show promise for targeted drug delivery to other tissues and cell types.

In addition to using the inherent properties of EVs or BNVs isolated from unmodified biological sources, groups have also directly or indirectly modified EVs to express specific proteins to achieve targeted delivery of chemotherapeutic drugs. For example, one group recently employed EVs modified to express a HER2 affibody-LAMP2-EGFP fusion protein to target the efficient delivery of miR-21i and 5-FU to colon cancer cells[99]. These proteins were chosen because LAMP2 was abundantly expressed on the EV surface, HER2 promoted EV targeting to colon cancer cells, and EGFP enhanced EV uptake. EVs were loaded with both 5-FU and miR-21i, an inhibitor of 5-FU resistance, to enhance the therapeutic effect of these modified EVs. This design was found to facilitate EV uptake by colon cancer cells, enhance 5-FU cytotoxicity in 5-FU-resistant colon cancer cells, and did not cause toxicity in the hematological system and major organs in mice models.

5.2. *Cancer vaccine platforms*

EVs can also carry tumor-associated antigens (TAAs) and may therefore serve as strong candidates for antigenic factors in cancer vaccines. EVs can have desirable properties as vaccine agents, since they can carry specific antigens expressed by their parental cells, and be effectively modified to adjust their immunogenic properties, including any adverse immunosuppressive associated with their parental cells. EVs also retain functional stability after short-term storage at 4 °C and thawing from frozen storage. EV RNA and protein levels and uptake efficiency were reported to be stable for seven, five, and 3 day at 4 °C, respectively, with EV uptake efficiency remaining stable for at least 7 days at −20 °C and 14 days and −80 °C[100]. Several currently available techniques can also be employed to protect the bioactivity of EV-based vaccines, including freezing, spray drying, and freeze-drying, which should facilitate the transportation, storage, and routine handling of EVs vaccines for clinical applications[101].

Studies now indicate that EVs derived from DCs and tumor cells can serve as vaccines for cancer immunotherapy[102,103]. EVs derived from DCs, the most potent professional APCs, can transfer peptide-MHC complexes to the plasma membranes of recipient DCs to evoke T cell activation responses[21,104]. One study engineered DCs to secrete EVs that abundantly expressed the liver protein α-fetoprotein (AFP EVs) to induce strong antigen-specific immune responses to attenuate tumor growth and prolong survival in mice with hepatocellular cancers revealing antigenic and pathological heterogeneity (Fig. 3B). Mice treated with AFP EVs had increased levels of $CD8^+$ T cell proliferation and IFN-γ and IL-2 and decreased levels of regulatory T (T_{reg}) cells and IL-10 and TGF-β in their tumors, indicating a potent effect to remodel their TME immune cell compositions. EVs secreted by tumor cells can natively express antigenic epitopes that stimulate cells to promote anti-tumor-specific immune responses[105]. However, modified EVs can function as adjuvants, materials used in most vaccines to enhance the immune response to their target antigen(s), and which play a critical role in effective presentation of tumor antigens in cancer vaccines[106]. Melanoma-derived EVs engineered to simultaneously deliver antigen and adjuvant to recipient DCs have been used to enhance their ability to achieve an effective cancer immunotherapy response. Melanoma cells were modified to express streptavidin fused to EV-trophic lactadherin to produce EVs that expressed streptavidin on their outer membrane (SAV-EVs). These SAV-EVs were then incubated with biotinylated CpG DNA to produce CpG-SAV-EVs that delivered tumor antigens and CpG adjuvant to recipient DCs. Notably, CpG-SAV-EV immunization increased DC activation, tumor antigen presentation, and anti-tumor effects, and mouse survival time more than co-administration EVs and CpG DNA[107] (Fig. 3C).

Bacterial outer membrane vesicles (OMVs), the bacterial analogs of EVs described in the "Infectious Disease Platform" section of this review, that exhibit native adjuvant activity can also be engineered to express tumor-specific antigens to induce strong immune responses required for effective cancer vaccine applications[108,109]. For example, one group modified OMVs to express basic fibroblast growth factor (BFGF), which has multiple functions to promote cancer cell survival, proliferation, invasion and tumor growth, and found that mice immunized with these modified OMVs developed a persistent anti-BFGF auto-antibody response that antagonized these pro-tumorigenic effects and induced tumor regression[110]. Another group recently described a versatile Plug-and-Display OMV-based vaccine platform that permits OMVs modified with two distinct "catcher" proteins to be subsequently modified with a variety of proteins. In this approach, proteins modified by either of two specific peptide tags are specifically recognized by their complementary catcher protein to spontaneously form an isopeptide bond, allowing OMVs multiple tumor-specific antigens to be rapidly attached to the surface catcher-modified OMVs at the same time by a by a simple incubation step[111,112] (Fig. 3D). Mice injected with melanoma cells and then vaccinated with catcher-decorated OMVs displaying melanoma-associated TRP2 protein demonstrated a near complete attenuation of lung metastasis in conjunction with robust indication of a tumor-specific immune response[111]. A similar response was also observed in different mouse metastasis model, although metastasis reductions observed with OMVs displaying each of the two analyzed single tumor antigens were less pronounced than those observed with OMVs displaying both antigens[111].

Notably, this OMV platform provides a flexible and convenient means to generate cancer vaccines by addition of tagged cancer-specific proteins to a standard preformulated OMV stock material. The ability to rapidly produce monovalent or multivalent cancer vaccines using this approach has major implications for the development of personalized cancer vaccines. This Plug-and-Display approach also has strong potential utility for OMV-based infectious disease vaccines, and for therapeutics that employ human EVs.

5.3. *Infectious disease vaccine platforms*

Emerging and re-emerging infectious diseases are constant threats to human health and remain a massive burden in some developing countries, but drug resistance and pathogen variations can hinder disease treatment and containment efforts. Vaccination is the most cost-effective and practical public health intervention to prevent and control infectious disease.

Vaccination is also a vital measure to produce herd immunity to infectious diseases that are subject to global outbreaks that can lead to epidemics and pandemics, and it is essential that such vaccines are designed in a manner that allows their rapid development and production. Several EVs properties, including their stability and potential cell/tissue targeting abilities, are of great interest to researchers developing new delivery vehicles for a variety of therapeutic agents. However, the ability of EVs to elicit protective immune responses is also of substantial interest for new vaccines to a spectrum of infectious diseases caused by bacterial, fungi, viral, and parasitic human pathogens[113–115], although most effort has focused on the use of OMVs for bacterial vaccines.

Similar to eukaryotic cells, bacteria spontaneously release membrane-defined vesicles (OMVs) into their extracellular environment through an outward membrane budding process[116]. Most OMV studies have used OMVs from Gram-negative bacteria since it is not clear how OMVs penetrate the thick cell walls of Gram-positive bacteria OMVs[117,118], and the mechanism(s) responsible for OMV secretion is not clear even in Gram-negative bacteria[119]. OMVs are spherical 20−250 nm diameter vesicles that are bounded by a lipid bilayer that contains lipopolysaccharide (LPS) and membrane proteins and which carry a variety of bioactive molecules, including DNA, RNAs, proteins, and peptidoglycan from the periplasm and cytoplasm[120] (Fig. 4), including factors that can induce protective immune responses[121].

OMVs have several properties that allow them to function as excellent adjuvants, including their vesicular structure and size, which allows easy entry the lymphatic system, and their transport of multiple pathogen-associated molecular patterns (PAMPs), including LPS, CpG rich DNA, peptidoglycan, and others that can stimulate potent immune responses[109]. Co-injection of OMVs with an antigen (*e.g.*, ovalbumin) can induce more robust innate and adaptive immune responses than co-administering antigen with CpG DNA or aluminum hydroxide adjuvants[2]. OMVs are promising vaccine platforms, particularly for microbial infections, with a comparison of OMV, whole-cell, and acellular vaccines for *Bordetella pertussis* infection found that the OMV vaccine produced a broad humoral response and the highest antibody titers[122,123].

Several potential issues should be considered when using bacterial OMVs, however, including their antigen repertoire their potential to induce LPS toxicity and other side effects. OMVs isolated from bacteria that express some variants of LPS can induce pyroptosis that can lead to sepsis at high concentration[2], while OMVs derived from a bacterial strain that expresses a less toxic form of LPS were more effecting in activating DCs than heat-inactivated or live-attenuated bacteria. OMVs can also be bioengineered for greater biocompatibility, lower toxicity, and to alter their interactions with the immune system[124]. For example, one study that engineered *Escherichia coli* BL21 to express only the biosynthetic precursor of LPS to reduce OMV toxicity, found that its OMVs still stimulated toll-like receptor four signaling to

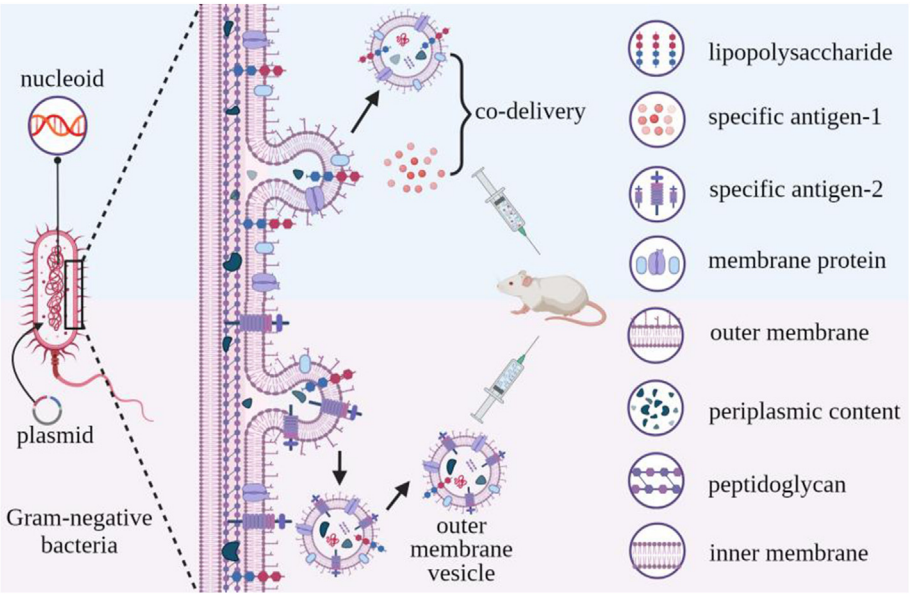


Figure 4 OMV formation, structure, and function. Gram-negative bacteria secrete OMVs by outward budding of their outer membrane. OMV potentially antigenic or pro-inflammatory factors, including their surface lipopolysaccharide and membrane proteins, and luminal cargoes of DNA, RNA, peptidoglycan and others factors, allowing them to serve as adjuvants that can be engineered display specific antigens and serve an antigen/adjuvant co-delivery platform for vaccine development.

function as adjuvants and revealed strong immunogenicity and low toxicity while producing a balanced Th1/Th2 humoral immune response[125]. Modification of these OMVs to link an influenza A-derived peptide to the OMV membrane protein ClyA also provided complete protection against influenza-induced mortality when three mouse strains were immunized with these OMVs prior to a challenge with a lethal dose of influenza A[125].

OMVs also represent a convenient means for delivery of polysaccharides employed in vaccines. Polysaccharides can represent attractive vaccine substrates but their isolation and conjugation processes can be complex, time-consuming, low yield, and expensive when applied to either natural or chemically synthesized material[126]. For example, poly-*N*-acetyl-D-glucosamine (PNAG) is a promising vaccine target generated by bacterial, fungal, and protozoan cells, but to achieve effective immunogenicity PNAG glycoform variants must be conjugated to a protein carrier[127]. However, antibodies generated to PNAG variants exhibit poor microbial killing and *in vivo* protection, and while deacetylated PNAG (dPNAG) glycoform variants can stimulate protective immune responses[128] the extraction or chemical synthesis of dPNAG variants and their conjugation to carrier proteins can be difficult and expensive. These challenges can be addressed by bioengineering OMVs to carry targeted polysaccharide antigens, and plasmid transfer into non-pathogenic *E coli*. Strains have been used to produce glycosylated OMVs (glycOMVs) that carried recombinant PNAG (rPNAG) or dPNAG (rdPNAG) variants. Mice immunized with rdPNAG-glycOMVs had the highest specific antibody titers and the longest overall survival when challenged with a lethal dose of *Staphylococcus aureus* when compared to mice injected with PBS, empty OMVs, and rPNAG-glycOMVs[129]. Similar results were obtained in mice immunized with rdPNAG-glycOMVs and challenged with a lethal dose of *Francisella tularensis*, indicating that vaccination with rPNAG-glycOMVs had broad activity to eliminate pathogens that express PNAG.

However, relatively little research has been focused on the development of OMV vaccines, despite the successful use of an OMV vaccine for *Neisseria meningitidis* for more than three decades. First generation *N. meningitidis* OMV vaccines generated by extracting OMVs with detergents to remove LPS and decrease endotoxin activity were found to be effective against epidemic *N. meningitidis* outbreaks in Cuba, Norway, and New Zealand, demonstrating greater than 70% efficiency in these populations. The immunogenicity of these OMV vaccines relied upon an immunodominant antigen, PorA, that exhibits high strain-to-strain variability, and resulted in vaccine strain-specificity[130]. Subsequently, bacteria were bioengineered to produce OMVs vaccines in which multivalent OMVs display six different PorA subtypes, and evoked a strong humoral immune response and protective effect in a phase I trial[131]. Recently, studies have investigated the potential utility of *B. pertussis* OMV vaccines since current vaccines do not evoke the same immune response as infection, exhibit waning immunity, and provide individual protection without preventing transmission[132]. No clinical studies have yet been performed with *B. pertussis* OMV vaccine candidates, but mouse studies have demonstrated promising results that indicate that *B. pertussis* OMVs can produce protection similar to challenge with heat-killed *B. pertussis* bacilli, which persists for up to 9 months, but induce less pro-inflammatory cytokines to address the adverse inflammatory response encountered with whole-cell vaccination.

OMVs have also been studied as vaccines for endemic and emerging human viruses, including influenza A H1N1, MERS-CoV, and Zika[133], including a multivalent vaccine for both Influenza A H1N1 Virus and MERS-CoV[134]. A SARS-CoV-2 OMV could also be developed using a similar approach, and the ability to rapidly modify some OMV vaccine platforms could be particularly useful when developing new vaccines to emerging SAR-CoV-2 variants of concern[135]. However, most companies and research institutions developing COVID-19 vaccines have focused on protein, DNA, RNA, and viral vector vaccines[136], which can each have distinct challenges. Limitation of virus vaccines have been summarized above, and protein vaccines must retain the conformation of their target protein in the presence of adjuvant. Naked DNA and RNA vaccines frequently exhibit low *in vivo* stability and uptake, and other nucleic acid and protein delivery methods, including virus-like particles, nanoparticles, and liposomes, typically induce weak immune responses and require adjuvants to promote protective immune responses[137]. Characteristics of recent nucleic acid vaccines that employ liposomes are not well known, although current SARS-CoV-2 RNA vaccines require low-temperature storage prior to use, and have short windows of activity after being thawed for use. Notably, however, most of these limitations do not apply to OMV vaccines, as discussed above.

In summary, OMVs can function as excellent antigen/adjuvant co-delivery vehicles that can directly activate the strong innate immune response without the safety risks associated with heat-inactivated or live-attenuated pathogens, or diseased cells. Bioengineering also allows streamlined construction of OMVs that can both function as adjuvants and present multiple antigens (*e.g.*, polysaccharides, proteins, and nucleic acids) to serve as multivalent vaccines against an array of targeted pathogens or cancers[138].

5.4. *Regenerative medicine*

Regenerative medicine applications attempt to repair or regenerate damaged, diseased or missing cells, tissues and organs to restore normal function. Mesenchymal stem/stromal cells (MSC)s are of great interest for regenerative medicine applications since they can differentiate to multiple lineages, self-renew, modulate immune responses, and can be isolated and cultured from multiple sources, cell lines, bone marrow, umbilical cord and adipose tissue, and others[139]. Many MSC effects can be attributed to paracrine signaling mechanisms where EVs function as key effectors[140]. Similar to MSCs, MSC-derived EVs (MSC-EVs) can deliver proteins, nucleic acids, and signaling molecules that maintain pluripotency, induce regenerative phenotypes, inhibit apoptotic reactions, and regulate immune responses, to promote regenerative repair of wounded cells and tissues[140]. However, unlike MSCs, MSC-EVs have simple storage and handling requirements, are less immunogenic, readily cross biological barriers due to their small size, and have fewer safety concerns (*e.g.*, cannot self-replicate and thus are not associated with a risk of neoplastic transformation)[141,142]. MSC-EVs represent a powerful tool to repair tissue damage associated with several chronic or degenerative diseases that can affect brain, heart, liver, lung, kidney, skin, and bone[143–145] (Fig. 5).

Recent MSC-EVs applications have shown great feasibility for regenerative medicine approaches intended to repair other tissue injuries. For example, surgical open-chest procedures used to place cardiac patches that transfer therapeutics to cardiac injury sites cause substantial trauma and adverse impacts. Recently, however, a minimally invasive procedure that sprays MSC-EVs and a U.S. Food and Drug Administration (FDA)-approved fibrin

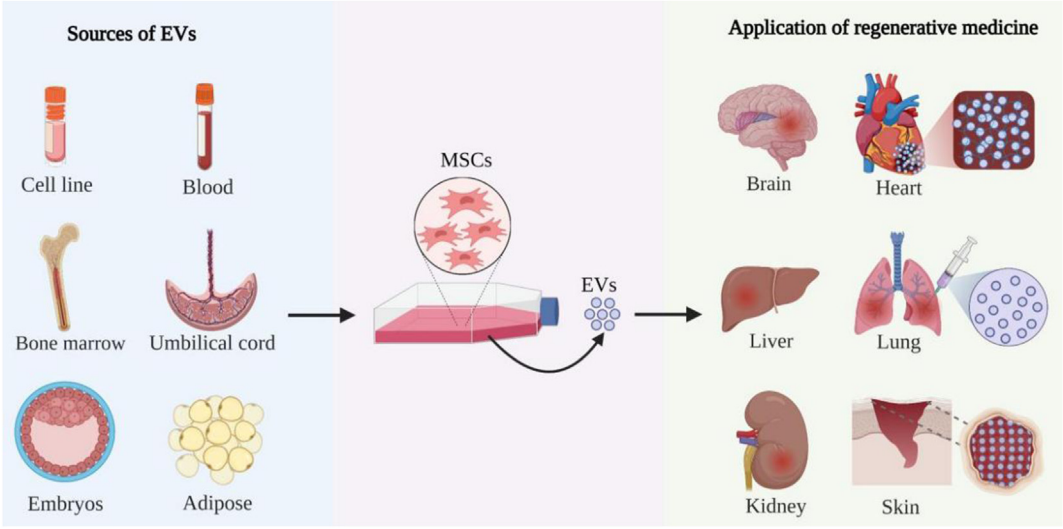


Figure 5 MSC-EV applications in regenerative medicine. MSCs obtained from commercial cell lines, bone marrow, blood, umbilical cord, embryonic, and adipose tissue can be cultured to isolate EVs to repair cell and tissue damage in the brain, heart, liver, lung, kidney, and skin. Examples cited in this review include the use of MSC-EVs to regenerate cardiomyocytes damaged after myocardial infarction, repair lung tissue damaged by pneumonia, ARDS, acute lung injury, or pulmonary fibrosis, or promote the repair large skin wounds in animal models.

scaffold directly onto the heart through a small thoracic incision has been reported to repair and regenerate damaged cardiac tissue[146]. Notably, this MSCs-EVs spray method extended MSC-EV retention, enhanced their cardiomyocyte uptake, improved cardiac function, enhanced angiomyogenesis, and diminished the infarct size to support cardiac regeneration.

MSC-EVs have recently been evaluated for treatment of COVID-19 associated tissue damage. Some individuals with severe SARS-CoV-2 infections develop diffused alveolar injury, endothelial cell damage, and bilateral interstitial pneumonia, which can lead to pulmonary fibrosis and acute respiratory distress syndrome (ARDS)[147]. New treatments are thus needed treat or repair this tissue damage. Multiple clinical trials have been registered (https://www.clinicaltrials.gov/) to investigate the safety and efficacy of MSCs-EV therapies for this purpose, but only one has been published to date. This trial reported that severe COVID-19 patients who received a single intravenous dose of MSCs-EVs revealed clinical and oxygenation status improvements without treatment-associated mortality or safety concerns during the 14-day post-treatment evaluation interval[148]. Further studies are needed to validate the safety and utility of MSC-EV therapy for severe COVID-19 cases, but the results of this study agree with the results of a systematic review of 39 studies that examined the use of EV treatments in animal models of pneumonia, ARDS, acute lung injury, or pulmonary fibrosis[149]. This review concluded that EV therapy had multiple beneficial effects to attenuate tissue damage and prolong survival, which included attenuating inflammation, promoting the repair of damaged alveolar and microvascular endothelial tissue, and attenuating or preventing pulmonary fibrosis. MSC-EVs thus appear likely to have great therapeutic to attenuate and repair COVID-19-related lung injuries by enhancing tissue regeneration via multiple pathways, which could greatly improve the current treatment landscape.

Cell-free, EV-laden biomaterial scaffolds are another area of growing interest in regenerative medicine [150], since some of these scaffolds are reported to regulate immune responses that promote tissue regeneration. One study found that immune cells were primarily responsible for *in vivo* uptake of scaffold-associated EVs, with the EVs and the scaffold matrix respectively functioning to recruit and train immune cells and synergistically induce macrophage and regulatory T cell responses to repair mouse severe skin wounds that would not otherwise heal[151]. Negatively charged MSCs-EVs were immobilized onto a positively charged fibrous polyester matrix by electrostatic interaction to prolong EV retention and allow the continuous uptake of these EVs by immune cells recruited to the injury site. This MSCs-EV uptake accelerated M2 macrophage polarization associated with tissue repair, partially by activating CD_4^+ T helper two cells (T_H2) and regulatory T cells (T_{reg}) to secrete cytokines and growth factors that favored M2 macrophage polarization.

5.5. *EVs as carriers for gene therapy agents*

Gene therapy offers the potential to resolve or attenuate otherwise incurable chronic diseases by *in situ* correction of a defective gene responsible for the pathology. Considerable effort has, however, focused on the development of safe gene delivery vectors since one of the first gene therapy patients died from a toxic response to the therapeutic vector. Both viral and non-viral vehicles are employed for gene delivery, and each has advantages and disadvantages. Virus-based gene delivery approaches primarily employ adenoviruses, adeno-associated viruses, lentiviruses, and retroviruses that have high loading and transfer efficiency, but which cannot fully escape immune surveillance, are expensive to employ, and can have significant safety risks[152,153]. Non-viral vectors use synthetic materials that can generally have lower safety risks, but have can have poor loading and targeting efficiency and may still have relatively high immunogenicity[154].

EVs of considerable interest as gene delivery vehicles since they play important roles in cell-to-cell communication events and therefore have high biocompatibility, low immunogenicity, can protect their cargoes from degradation in the extracellular space and circulation, and target specific cells or tissues. EV safety

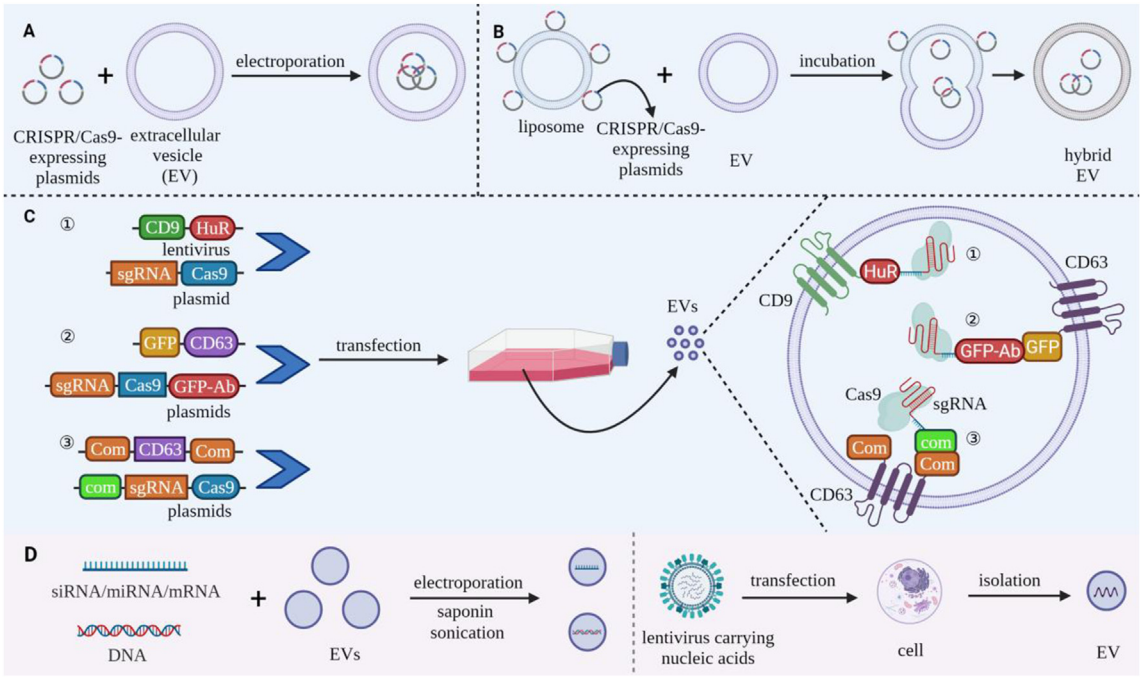


Figure 6 Strategies for loading bioactive components into EVs for gene therapy. (A−C) Load approaches for CRISPR/Cas9 gene editing systems. (A) Electroporation-mediate loading of Cas9/sgRNA-expressing plasmids into large-diameter EVs. (B) EV fusion with liposomes that are surface loaded with Cas9/sgRNA-expressing by electrostatic interaction, which transfers these plasmids to the lumen of the resulting hybrid EVs. (C) Transfection of parental cells with vectors that express fusion proteins that induce the EV enrichment of recombinant Cas9/sgRNA complexes. Approaches reported to date include: ① CD9-HuR fusion protein-mediated capture of CRISPR/Cas9 complexes containing an miR-155-tagged sgRNA. ② CD63-GFP fusion-protein mediated capture of CRISPR/Cas9 complexes containing a Cas9-GFP nanobody fusion protein. ③ CD63-com fusion protein capture of CRISPR/Cas9 complexes that contain sgRNA modified with the com aptamer (D) EV loading with nucleic acids (*e.g.*, siRNA, miRNA, mRNA, and DNA). By electroporation, sonication, or saponin-mediated membrane permeation, or during EV biogenesis in parental cells following lentivirus transfection.

profiles are also better than viruses, since they cannot replicate and their composition can be controlled by careful selection or genetic modification of their parental cells. Gene engineering or other modification approaches can also be used to introduce new therapeutic cargoes or alter their target them to specific cells or tissues. Recent studies have shown exciting progress in using EVs to deliver CRISPR/Cas complexes for *in situ* gene editing as well as, and RNA and DNA cargoes with therapeutic activity.

5.5.1. EV delivery of CRISPR/Cas9 therapies

CRISPR/Cas9 complexes contain Cas9 protein and a single guide RNA (sgRNA) that recognizes a complementary DNA sequence, causing Cas9 to efficiently bind and cleave this DNA target for precise gene editing[155]. However, CRISPR/Cas9 gene therapy approaches require efficient delivery of CRISPR/Cas9 complexes into targeted cells, and current delivery vehicles exhibit relatively high immunogenicity and poor transfer efficiency and specificity[156]. EV electroporation has been used to package CRISPR/Cas9-expressing plasmids into EVs for delivery to ovarian cancer tumors in SKOV3 xenograft mice (Fig. 6A). EVs derived from cancer cells were more effective in mediating genome editing in these tumors than those derived from epithelial cells in this study, likely due to the tropism of the cancer-derived EVs, although the use of tumor-derived EVs raises potential safety concerns[157]. Directly loading EVs with a CRISPR/Cas9 plasmid by electroporation or sonication was not useful, since small EVs (50−150 nm diameter) were not effective carriers due to their limited volume and larger EVs that could encapsulate the CRISPR/Cas9 plasmids had low loading efficiency. However, hybrid EVs, produced by fusing EVs with liposomes, can package large nucleic acid molecules to address this issue[158] (Fig. 6B). Hybrid EVs have been loaded with CRISPR/Cas9 expression plasmid by mixing negatively charged EVs with positively charged liposomes that have bound plasmid DNA *via* electrostatic interaction[159], since subsequent incubation of these vesicles induced their fusion. This hybrid EV approach successfully delivered CRISPR/Cas9 plasmids into MSCs that were not effectively transfected by liposomes[159].

Bioengineering can also produce modified EVs that promote the delivery of gene cargoes. For example, CRISPR/Cas9 components have been enriched in EV cargoes (Fig. 6C) by fusing EV membrane proteins with proteins that bound specific tags. In one study, the EV membrane protein CD9 was fused with HuR, an RNA binding protein with high affinity for miR-155, to enrich recombinant Cas9 mRNA and sgRNA transcripts tagged with miR-155 sequence. Cells were transfected with vectors expressing miR-155-tagged sgRNA and Cas9 transcripts and CD9-HuR. EVs produced by these cells were enriched in the tagged sgRNA and Cas9 transcripts and reduced both *in vitro* and *in vivo* expression of the sgRNA-targeted gene[160]. Similarly, vectors expressing the EV membrane protein CD63 fused with GFP and Cas9 fused with a GFP-specific nanobody were used to enhance EV enrichment of Cas9[161]. Recently, a vector expressing a recombinant CD63 protein modified at both termini with the aptamer binding protein

Table 3 Representative examples of EVs used as therapeutic agent carriers.

Application	Therapeutic agent	Donor cell	Loading strategy	Advantage	Ref.
Cancer therapy	PTX	Cow milk	Incubation	Exhibiting low systemic toxicity and excellent stability	98
	5-FU, miR-21i and Her2 affibody-LAMP2-EGFP	Colorectal cancer ($HCT\text{-}116^{5FR}$)	Electroporation	Targeting cancer cells overexpressing Her2; Facilitating cellular uptake and improving the cytotoxicity for 5-FU-resistant cells	99
	AFP antigen	Dendritic cells	Lentivirus	Disseminating antigenic material among DCs	105
	Tumor specific antigen and CpG DNA adjuvant	Melanoma (B16)	Incubation	Delivering CpG-EVs tumor specific antigen; Exerting stronger anti-tumor effects than co-delivery	107
	BFGF antigen	*E. coli*	Transfecion (plasmid)	Producing persistent anti-BFGF auto-antibodies	110
	DOX	Glioblastoma (bEnd.3)	Sonication	Crossing the BBB; Escaping lysosomal degradation; Low cytotoxicity and exhibiting excellent tumor suppression effect	175
Infectious disease vaccine	Adjuvant	*B. pseudomallei* (strain Bp82)	–	Low toxicity and strong immunostimulation	2
	The specific antigen peptide	*B. pertussis* (strain B1917)	–	Eliciting high antibody level and inducing broad humoral response	122,123
	Lipid IVa instead of full LPS	*E. coli*	Transduction (phage)	Serving as the adjuvant to show high immunogenicity and low toxicity	125
	dPNAG polysaccharide antigen	*E. coli* (strain BL21)	–	Broadly eliminating pathogens expressing PNAG on the surface	129
	PorA *N. meningitidis* antigen	*N. meningitidis* (strain PL16215 or PL10124)	–	Evoking strong humoral immune response and produce a powerful protective effect	131
Regenerative medicine	Proteins and nucleic acids with repaired and regenerative functions	Mesenchymal Stem cells (main)	–	Enhance lung tissue regeneration in multiple pathways	150
	Proteins and nucleic acids with repaired and regenerative Functions, fibrinogen and thrombin	Mesenchymal stem cells	Co-delivery	Extending the retention and promote uptake of EVs	151
	Proteins and nucleic acids with repaired and regenerative functions, fibrous polyester materials	Mesenchymal stem cells	Incubation	Prolong the retention of EVs; Recruit and active uptake EVs of immune cells	151

(*continued on next page*)

Table 3 (*continued*)

Application	Therapeutic agent	Donor cell	Loading strategy	Advantage	Ref.
Gene therapy	CRISPR/Cas9	Ovarian cancer (SKOV3) IVA	Electroporation (CRISPR/Cas9-expressing plasmid)	Achieving to load large molecule nucleic acids; CRISPR/Cas9 selectively accumulate in cancer cell	157
	CRISPR/Cas9	293T	Incubation (EVs fuse with liposome carrying CRISPR/Cas9-expressing plasmid)	Higher loading efficiency than electroporation	158
	CRISPR/Cas9	293T	Transfection (CD9-HuR (plasmid) and sgRNA-Cas9 (lentivirus))	Improving gene editing efficiency, safety and flexibility	160
	CRISPR/Cas9	293T	Transfection (GFP-CD63 (plasmid) and sgRNA-Cas9-GFP Ab (plasmid)	Improving gene editing efficiency, safety and flexibility	161
	CRISPR/Cas9	293T	Transfection (com-sgRNA (plasmid), Com-CD63-Com (plasmid) and Cas9-com (plasmid))	Improving gene editing efficiency, safety and flexibility	162
	miR-31	293T	Transfection (lentivirus)	Promoting the wound healing; safety	164
	miRNA (Let-7i, miR-142 and miR-155)	Breast cancer (41T)	Electroporation	Modulating immune response and tumor microenvironment to reduce tumor burden	165
	Neuron-specific RVG peptide and miRNA	Not available	Electroporation	Crossing BBB, target specific cells	166
	siRNA	Pancreatic cancer (PANC-1)	Electroporation	Lower toxicity and equal treatment efficiency comparing with transfection reagent	169
	Low-density lipoprotein receptor (Ldlr) mRNA	Liver cell (AML12)	Transfection (plasmid)	Mainly targeting the liver then producing ample Ldlr protein	170
	DNA	Not available	Sonication or saponin	High loading efficiency	171
	Linear DNA	293T or HUVEC	Electroporation	Every large size EV contains hundreds of DNA	172

com was also used to promote EV enrichment of CRISPR/Cas9 ribonucleotide complexes containing an sgRNA that was tagged with the com aptamer to efficient gene editing[162].

5.5.2. *RNA-based therapies*

RNA-based therapeutics have primarily relied on the delivery of miRNA, siRNA, or mRNA cargoes that directly or indirectly inhibit the activity of a defective or dysfunctional gene or restore the normal activity or a target gene. EVs are gaining popularity for these approaches since RNA cargoes are protected from degradation, can traverse biological barriers, and be targeted to specific cell types. Common methods of RNA transfer are summarized in Fig. 6D. Recent EV preclinical studies have primarily focused on the delivery of miRNAs therapeutics to attenuate a diverse array of diseases, although such approaches may lack specificity and have off-target effects since any given miRNA may regulate hundreds of mRNAs[163].

For example, EVs engineered to express miR-31 are reported to inhibit the expression of hypoxia-inducible factor 1-alpha inhibitor (HIF1AN) and epithelial membrane protein-1 (EMP-1) to enhance wound healing in diabetic Sprague Dawley rats by promoting angiogenesis, fibrogenesis, and re-epithelization[164]. Intramuscular injection of tumor-derived EVs loaded with three miRNAs (Let-7i, miR-142, and miR-155), which enhanced *in vitro* DC maturation and T cell proliferation and cytotoxicity, was also found to decrease tumor growth and increase survival in a mouse breast cancer model, which was associated with a shift from poorly differentiated to well-differentiated tumor phenotypes and an increase in tumor cell necrosis[165].

Similar studies have been performed with siRNAs, short synthetic RNAs designed to bind a complementary RNA sequence specific for a unique mRNA target. For example, one study used genetic engineering to fuse a neuron-specific RVG peptide to the EV membrane protein LAMP and employed electroporation to load these EVs with a siRNA specific for beta-secretase 1 (BACE1), a therapeutic target in Alzheimer's disease. These modified EVs were found to efficiently cross the BBB after intravenous injection into a mouse model of Alzheimer's Disease, resulting in ~60% less BACE1 mRNA and protein in the brains of these mice[166]. Similarly, injecting tumors in a mouse model of pancreatic cancer with EVs loaded with a siRNA specific for PAK4, whose overexpression promotes cell proliferation, migration and invasion[167,168], decreased tumor growth and increased mouse survival corresponding to tumor PAK4 decreases and necrosis increases[169].

EVs have also been employed to deliver mRNAs to induce therapeutic protein expression in targeted recipient cells. For example, intravenous injection of low-density lipoprotein receptor (Ldlr) deficient mice with EVs loaded with *Ldlr* mRNA restored liver Ldlr protein expression reduce liver lipid deposition and pro-inflammatory and pro-fibrotic gene expression and decrease atherosclerotic plaque formation following a high-fat diet challenge[170].

5.5.3. *DNA-based therapies*

Relatively few studies have examined the use of EVs to package and deliver DNA *versus* RNA to target cells[171], and efficient DNA loading into EVs appears to be a limiting factor in the development of therapeutics that rely upon DNA expression vectors. DNA and length and EV volume are reported to limit EV loading by electroporation, with short linear DNA (<1000 bp) being more efficiency loaded than longer linear or circular DNAs, and exosome-like EVs exhibiting reduced loading capacities than larger microvesicle-like EVs[172]. DNA-loaded EVs produced in this study were found to transfer their DNA cargoes to recipient cells, but gene expression was not observed following DNA transfer. It may thus be necessary to carefully refine electroporation conditions since this process may promote EV aggregation and changes in EV morphology that could affect the recovery of functional EVs.

Sonication has been used to load small DNA fragments into EVs with high loading efficiency, but these EVs have not shown encouraging therapeutic effects *in vivo*[171], likely due to disruption of EV integrity during the loading process[173]. EV treatment with surfactant saponin reagents has been used to increase EV membrane permeability without destroying the lipid bilayer structure and may be useful in DNA loading, but few studies have examined the ability of saponin reagents to load DNA into EVs. Further, given its hemolytic activity, saponin concentrations should be kept low and residual saponin should be removed from the loaded EVs[174]. Finally, for all these methods, care must be taken in selecting an appropriate separation strategy following the DNA loading procedure, since EVs may co-precipitate with unincorporated DNA to compromise DNA loading estimates.

6. Challenges and future perspectives

We have reviewed how these EV properties have been employed in EV applications with representative examples provided in Table 3. EVs research has made great progress in since EVs were first described 30 years ago, but further work needs to be done to address remaining challenges that can limit the development and use of EV-based clinical applications. Studies have defined the basic processes involved in EV biogenesis and some of the mechanisms that regulate their participation in cell−cell communication, but additional studies are required to clarify the exact mechanisms involved and if this information can be used to promote selective biogenesis, secretion, or isolation of desired EV subsets. Several EV features must also be considered when designing an EV-based therapeutic application.

EV selection is a critical factor in all EV applications, since different EVs may be appropriate for different therapeutic applications, and multiple factors can influence the selection of the EV source, including the inherent and/or engineered targeting and regulatory properties of the final EVs, their relative immunogenicity, and their ease of production or isolation. EVs secreted by primary cells or cells lines may natively express desired regulatory factors but lack target specificity, if required, or selectively target a desired cell population but lack desired therapeutic activity, and thus require manipulation thorough genetic engineering or other approaches to confer necessary properties or attenuate undesired activities. Scale and reproducibility considerations may also influence the selection of native or synthetic EV production methods.

For example, EVs derived from immune cells, and particularly DCs, are frequently used for cancer therapeutics since they can confer unique immunomodulatory properties to recipient tumor cells. EV-based vaccines usually employ EVs that carry, or can be modified to carry, both antigens and adjuvants. OMVs are an attractive choice but may require modification to avoid excessive inflammation. Tumor-derived EVs are rarely used in most EV applications, including cancer vaccines, due to safety concerns that these EVs might transfer factors that could promote tumor growth and the establishment of pre-metastatic niches. However,

EV derived from microbial pathogens, which naturally express both pathogen-specific antigens and factors that can serve as adjuvants, have been used in vaccines against their source organisms. Finally, EVs secreted by mesenchymal stem cells (MSCs) are frequently used in regenerative medicine since their cargoes have been reported to promote tissue repair and exert anti-inflammatory effects.

Different EV isolation strategies may also affect their purity, subtype enrichment and structural integrity of EV samples. Further study is thus required to define how EV isolation methods and cell culture conditions, viability, differentiation state, or other factors, influence the isolation of EV subtypes, and their integrity, surface markers, cargoes for specific applications. Standard culture conditions and precise isolation procedures defined in such studies are needed to produce stable, reproducible, and high purity EV isolates with consistent functional activities. Scale-up studies will also be required to validate that change in culture and isolation volumes do not alter EV characteristics. Further studies will also be required to evaluate if drug loading strategies or bioengineering approaches alter the original characteristics of these EVs and whether additional modifications are required to reduce their immunogenicity or toxicity prior to their translation into clinical applications.

Finally, different drug loading and surface modification approaches can influence EV loading capacity and integrity to alter EV-based drug delivery. It is therefore important to optimize both EV loading procedures for different therapeutic agents and EV modification approaches used to target specific cell and tissue targets to maximize the bioavailability of EV therapeutics at desired sites and to reduce therapeutic doses and systemic side effects.

Acknowledgments

This work was supported by Tulane Weatherhead Endowment Fund (USA). All figures were created with BioRender.com.

Author contributions

Shan Liu and Xue Wu searched the literatures and wrote the manuscript. Chandra Sutapa and Christopher Lyon edited the language and provided the new idea. Bo Ning provided the idea and drew the figures and tables. Li Jiang and Jia Fan drew the figures. Tony Y. Hu conceived the study and provided the guidance of the whole study.

Conflicts of interest

The authors have no conflicts of interest to declare.

References

1. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol* 2002;**2**:569−79.
2. Prior JT, Davitt C, Kurtz J, Gellings P, McLachlan JB, Morici LA. Bacterial-derived outer membrane vesicles are potent adjuvants that drive humoral and cellular immune responses. *Pharmaceutics* 2021;**13**:131.
3. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem* 1987;**262**:9412−20.
4. Armstrong JPK, Holme MN, Stevens MM. Re-engineering extracellular vesicles as smart nanoscale therapeutics. *ACS Nano* 2017;**11**:69−83.
5. Guo Q, Jiang C. Delivery strategies for macromolecular drugs in cancer therapy. *Acta Pharm Sin B* 2020;**10**:979−86.
6. Cataldi M, Vigliotti C, Mosca T, Cammarota M, Capone D. Emerging role of the spleen in the pharmacokinetics of monoclonal antibodies, nanoparticles and exosomes. *Int J Mol Sci* 2017;**18**:1248.
7. Tian T, Zhang HX, He CP, Fan S, Zhu YL, Qi C, et al. Surface functionalized exosomes as targeted drug delivery vehicles for cerebral ischemia therapy. *Biomaterials* 2018;**150**:137−49.
8. Wang Y, Pang J, Wang Q, Yan L, Wang L, Xing Z, et al. Delivering antisense oligonucleotides across the blood-brain barrier by tumor cell-derived small apoptotic bodies. *Adv Sci (Weinh)* 2021;**8**:2004929.
9. Willms E, Cabañas C, Mäger I, Wood MJA, Vader P. Extracellular vesicle heterogeneity: subpopulations, isolation techniques, and diverse functions in cancer progression. *Front Immunol* 2018;**9**:738.
10. Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. *J Proteonomics* 2010;**73**:1907−20.
11. Rai A, Fang H, Claridge B, Simpson RJ, Greening DW. Proteomic dissection of large extracellular vesicle surfaceome unravels interactive surface platform. *J Extracell Vesicles* 2021;**10**:e12164.
12. Mathieu M, Nevo N, Jouve M, Valenzuela JI, Maurin M, Verweij FJ, et al. Specificities of exosome versus small ectosome secretion revealed by live intracellular tracking of CD63 and CD9. *Nat Commun* 2021;**12**:4389.
13. Zhang H, Freitas D, Kim HS, Fabijanic K, Li Z, Chen H, et al. Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. *Nat Cell Biol* 2018;**20**:332−43.
14. Yan W, Jiang S. Immune cell-derived exosomes in the cancer-immunity cycle. *Trends Cancer* 2020;**6**:506−17.
15. Jong AY, Wu CH, Li J, Sun J, Fabbri M, Wayne AS, et al. Large-scale isolation and cytotoxicity of extracellular vesicles derived from activated human natural killer cells. *J Extracell Vesicles* 2017;**6**:1294368.
16. Zhu L, Kalimuthu S, Gangadaran P, Oh JM, Lee HW, Baek SH, et al. Exosomes derived from natural killer cells exert therapeutic effect in melanoma. *Theranostics* 2017;**7**:2732−45.
17. Zhou J, Tan X, Tan Y, Li Q, Ma J, Wang G. Mesenchymal stem cell derived exosomes in cancer progression, metastasis and drug delivery: a comprehensive review. *J Cancer* 2018;**9**:3129−37.
18. Liu T, Zhu Y, Zhao R, Wei X, Xin X. Visualization of exosomes from mesenchymal stem cells *in vivo* by magnetic resonance imaging. *Magn Reson Imaging* 2020;**68**:75−82.
19. Wang J, Sun X, Zhao J, Yang Y, Cai X, Xu J, et al. Exosomes: a novel strategy for treatment and prevention of diseases. *Front Pharmacol* 2017;**8**:300.
20. He C, Zheng S, Luo Y, Wang B. Exosome theranostics: biology and translational medicine. *Theranostics* 2018;**8**:237−55.
21. Thery C, Duban L, Segura E, Veron P, Lantz O, Amigorena S. Indirect activation of naive $CD4^+$ T cells by dendritic cell-derived exosomes. *Nat Immunol* 2002;**3**:1156−62.
22. Chen G, Huang AC, Zhang W, Zhang G, Wu M, Xu W, et al. Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. *Nature* 2018;**560**:382−6.
23. Parolini I, Federici C, Raggi C, Lugini L, Palleschi S, De Milito A, et al. Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem* 2009;**284**:34211−22.
24. Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan ML, Karlsson JM, et al. Mechanism of transfer of functional micrornas between mouse dendritic cells *via* exosomes. *Blood* 2012;**119**:756−66.
25. Pironti G, Strachan RT, Abraham D, Mon-Wei Yu S, Chen M, Chen W, et al. Circulating exosomes induced by cardiac pressure

overload contain functional angiotensin II type 1 receptors. *Circulation* 2015;**131**:2120−30.
26. Mulcahy LA, Pink RC, Carter DR. Routes and mechanisms of extracellular vesicle uptake. *J Extracell Vesicles* 2014;**3**:10.3402.
27. Gurung S, Perocheau D, Touramanidou L, Baruteau J. The exosome journey: from biogenesis to uptake and intracellular signalling. *Cell Commun Signal* 2021;**19**:47.
28. Kiss AL, Botos E. Endocytosis *via* caveolae: alternative pathway with distinct cellular compartments to avoid lysosomal degradation?. *J Cell Mol Med* 2009;**13**:1228−37.
29. Ni K, Wang C, Carnino JM, Jin Y. The evolving role of Caveolin-1: a critical regulator of extracellular vesicles. *Med Sci (Basel)* 2020;**8**:46.
30. Nanbo A, Kawanishi E, Yoshida R, Yoshiyama H. Exosomes derived from Epstein-Barr virus-infected cells are internalized *via* caveola-dependent endocytosis and promote phenotypic modulation in target cells. *J Virol* 2013;**87**:10334−47.
31. Svensson KJ, Christianson HC, Wittrup A, Bourseau-Guilmain E, Lindqvist E, Svensson LM, et al. Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid raft-mediated endocytosis negatively regulated by caveolin-1. *J Biol Chem* 2013;**288**:17713−24.
32. Delenclos M, Trendafilova T, Mahesh D, Baine AM, Moussaud S, Yan IK, et al. Investigation of endocytic pathways for the internalization of exosome-associated oligomeric alpha-synuclein. *Front Neurosci* 2017;**11**:172.
33. Costa Verdera H, Gitz-Francois JJ, Schiffelers RM, Vader P. Cellular uptake of extracellular vesicles is mediated by clathrin-independent endocytosis and macropinocytosis. *J Control Release* 2017;**266**:100−8.
34. Gordon S. Phagocytosis: an immunobiologic process. *Immunity* 2016;**44**:463−75.
35. Zheng Y, Tu C, Zhang J, Wang J. Inhibition of multiple myeloma-derived exosomes uptake suppresses the functional response in bone marrow stromal cell. *Int J Oncol* 2019;**54**:1061−70.
36. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol* 2018;**19**:213−28.
37. Bissig C, Gruenberg J. Alix and the multivesicular endosome: alix in wonderland. *Trends Cell Biol* 2014;**24**:19−25.
38. Liu J, Jiang F, Jiang Y, Wang Y, Li Z, Shi X, et al. Roles of exosomes in ocular diseases. *Int J Nanomed* 2020;**15**:10519−38.
39. Vincent-Schneider H, Stumptner-Cuvelette P, Lankar D, Pain S, Raposo G, Benaroch P, et al. Exosomes bearing HLA-DR1 molecules need dendritic cells to efficiently stimulate specific t cells. *Int Immunol* 2002;**14**:713−22.
40. Bhatnagar S, Schorey JS. Exosomes released from infected macrophages contain mycobacterium avium glycopeptidolipids and are proinflammatory. *J Biol Chem* 2007;**282**:25779−89.
41. Menon R, Debnath C, Lai A, Guanzon D, Bhatnagar S, Kshetrapal PK, et al. Circulating exosomal miRNA profile during term and preterm birth pregnancies: a longitudinal study. *Endocrinology* 2019;**160**:249−75.
42. Zhang L, Jiao G, Ren S, Zhang X, Li C, Wu W, et al. Exosomes from bone marrow mesenchymal stem cells enhance fracture healing through the promotion of osteogenesis and angiogenesis in a rat model of nonunion. *Stem Cell Res Ther* 2020;**11**:38.
43. Johnson J, Wu YW, Blyth C, Lichtfuss G, Goubran H, Burnouf T. Prospective therapeutic applications of platelet extracellular vesicles. *Trends Biotechnol* 2021;**39**:598−612.
44. Wu X, Wang L, Cong M, Shen M, He Q, Ding F, et al. Extracellular vesicles from skin precursor-derived schwann cells promote axonal outgrowth and regeneration of motoneurons *via* Akt/mTOR/p70S6K pathway. *Ann Transl Med* 2020;**8**:1640.
45. Zhang L, Yu D. Exosomes in cancer development, metastasis, and immunity. *Biochim Biophys Acta Rev Cancer* 2019;**1871**:455−68.
46. Li I, Nabet BY. Exosomes in the tumor microenvironment as mediators of cancer therapy resistance. *Mol Cancer* 2019;**18**:32.
47. Yang E, Wang X, Gong Z, Yu M, Wu H, Zhang D. Exosome-mediated metabolic reprogramming: the emerging role in tumor microenvironment remodeling and its influence on cancer progression. *Signal Transduct Targeted Ther* 2020;**5**:242.
48. Chen Q, Li Y, Liu Y, Xu W, Zhu X. Exosomal non-coding RNAs-mediated crosstalk in the tumor microenvironment. *Front Cell Dev Biol* 2021;**9**:646864.
49. Tan S, Xia L, Yi P, Han Y, Tang L, Pan Q, et al. Exosomal miRNAs in tumor microenvironment. *J Exp Clin Cancer Res* 2020;**39**:67.
50. Neviani P, Wise PM, Murtadha M, Liu CW, Wu CH, Jong AY, et al. Natural killer-derived exosomal MIR-186 inhibits neuroblastoma growth and immune escape mechanisms. *Cancer Res* 2019;**79**:1151−64.
51. Beatriz M, Vilaca R, Lopes C. Exosomes: innocent bystanders or critical culprits in neurodegenerative diseases. *Front Cell Dev Biol* 2021;**9**:635104.
52. Pinnell JR, Cui M, Tieu K. Exosomes in Parkinson disease. *J Neurochem* 2021;**157**:413−28.
53. Guo M, Wang J, Zhao Y, Feng Y, Han S, Dong Q, et al. Microglial exosomes facilitate α-synuclein transmission in Parkinson's disease. *Brain* 2020;**143**:1476−97.
54. Zheng D, Huo M, Li B, Wang W, Piao H, Wang Y, et al. The role of exosomes and exosomal microrna in cardiovascular disease. *Front Cell Dev Biol* 2020;**8**:616161.
55. Lu Y, Liu D, Feng Q, Liu Z. Diabetic nephropathy: perspective on extracellular vesicles. *Front Immunol* 2020;**11**:943.
56. Giro O, Jimenez A, Pane A, Badimon L, Ortega E, Chiva-Blanch G. Extracellular vesicles in atherothrombosis and cardiovascular disease: friends and foes. *Atherosclerosis* 2021;**330**:61−75.
57. Meckes Jr DG, Shair KH, Marquitz AR, Kung CP, Edwards RH, Raab-Traub N. Human tumor virus utilizes exosomes for intercellular communication. *Proc Natl Acad Sci U S A* 2010;**107**:20370−5.
58. Zhang L, Ju Y, Chen S, Ren L. Recent progress on exosomes in RNA virus infection. *Viruses* 2021;**13**:256.
59. Bukong TN, Momen-Heravi F, Kodys K, Bala S, Szabo G. Exosomes from hepatitis C infected patients transmit HCV infection and contain replication competent viral RNA in complex with Ago2-miR122-HSP90. *PLoS Pathog* 2014;**10**:e1004424.
60. Chen TC, Hsieh CH, Sarnow P. Supporting role for GTPase Rab27a in hepatitis C virus RNA replication through a novel miR-122-mediated effect. *PLoS Pathog* 2015;**11**:e1005116.
61. Sole C, Moline T, Vidal M, Ordi-Ros J, Cortes-Hernandez J. An exosomal urinary miRNA signature for early diagnosis of renal fibrosis in lupus nephritis. *Cells* 2019;**8**:773.
62. Pawlak EN, Dikeakos JD. HIV-1 Nef: a master manipulator of the membrane trafficking machinery mediating immune evasion. *Biochim Biophys Acta* 2015;**1850**:733−41.
63. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in exosome isolation techniques. *Theranostics* 2017;**7**:789−804.
64. Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes *in vitro*: selective externalization of the receptor. *Cell* 1983;**33**:967−78.
65. Gandham S, Su X, Wood J, Nocera AL, Alli SC, Milane L, et al. Technologies and standardization in research on extracellular vesicles. *Trends Biotechnol* 2020;**38**:1066−98.
66. Gardiner C, Vizio DD, Sahoo S, Théry C, Witwer KW, Wauben M, et al. Techniques used for the isolation and characterization of extracellular vesicles: results of a worldwide survey. *J Extracell Vesicles* 2016;**5**:32945.
67. Bang C, Batkai S, Dangwal S, Gupta SK, Foinquinos A, Holzmann A, et al. Cardiac fibroblast-derived microrna passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy. *J Clin Invest* 2014;**124**:2136−46.
68. Müller D, Cattaneo S, Meier F, Welz R, de Mello AJ. Nanoparticle separation with a miniaturized asymmetrical flow field-flow fractionation cartridge. *Front Chem* 2015;**3**:45.

69. Taylor DD, Shah S. Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes. *Methods* 2015;**87**:3−10.
70. Böing AN, van der Pol E, Grootemaat AE, Coumans FA, Sturk A, Nieuwland R. Single-step isolation of extracellular vesicles by size-exclusion chromatography. *J Extracell Vesicles* 2014;**3**:23430.
71. Muller L, Hong CS, Stolz DB, Watkins SC, Whiteside TL. Isolation of biologically-active exosomes from human plasma. *J Immunol Methods* 2014;**411**:55−65.
72. Gámez-Valero A, Monguió-Tortajada M, Carreras-Planella L, Franquesa Ml, Beyer K, Borràs FE. Size-exclusion chromatography-based isolation minimally alters extracellular vesicles characteristics compared to precipitating agents. *Sci Rep* 2016;**6**:33641.
73. Deregibus MC, Figliolini F, D'Antico S, Manzini PM, Pasquino C, De Lena M, et al. Charge-based precipitation of extracellular vesicles. *Int J Mol Med* 2016;**38**:1359−66.
74. Weng Y, Sui Z, Shan Y, Hu Y, Chen Y, Zhang L, et al. Effective isolation of exosomes with polyethylene glycol from cell culture supernatant for in-depth proteome profiling. *Analyst* 2016;**141**:4640−6.
75. Zarovni N, Corrado A, Guazzi P, Zocco D, Lari E, Radano G, et al. Integrated isolation and quantitative analysis of exosome shuttled proteins and nucleic acids using immunocapture approaches. *Methods* 2015;**87**:46−58.
76. Tauro BJ, Greening DW, Mathias RA, Ji H, Mathivanan S, Scott AM, et al. Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes. *Methods* 2012;**56**:293−304.
77. Greening DW, Xu R, Ji H, Tauro BJ, Simpson RJ. A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. *Methods Mol Biol* 2015;**1295**:179−209.
78. Wang Z, Wu HJ, Fine D, Schmulen J, Hu Y, Godin B, et al. Ciliated micropillars for the microfluidic-based isolation of nanoscale lipid vesicles. *Lab Chip* 2013;**13**:2879−82.
79. Choi D, Montermini L, Jeong H, Sharma S, Meehan B, Rak J. Mapping subpopulations of cancer cell-derived extracellular vesicles and particles by nano-flow cytometry. *ACS Nano* 2019;**13**:10499−511.
80. Maguire CM, Rosslein M, Wick P, Prina-Mello A. Characterisation of particles in solution—a perspective on light scattering and comparative technologies. *Sci Technol Adv Mater* 2018;**19**:732−45.
81. Panagopoulou MS, Wark AW, Birch DJS, Gregory CD. Phenotypic analysis of extracellular vesicles: a review on the applications of fluorescence. *J Extracell Vesicles* 2020;**9**:1710020.
82. Coughlan C, Bruce KD, Burgy O, Boyd TD, Michel CR, Garcia-Perez JE, et al. Exosome isolation by ultracentrifugation and precipitation and techniques for downstream analyses. *Curr Protoc Cell Biol* 2020;**88**:e110.
83. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the international society for extracellular vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018;**7**:1535750.
84. Mehryab F, Rabbani S, Shahhosseini S, Shekari F, Fatahi Y, Baharvand H, et al. Exosomes as a next-generation drug delivery system: an update on drug loading approaches, characterization, and clinical application challenges. *Acta Biomater* 2020;**113**:42−62.
85. Li S, Yi M, Dong B, Tan X, Luo S, Wu K. The role of exosomes in liquid biopsy for cancer diagnosis and prognosis prediction. *Int J Cancer* 2021;**148**:2640−51.
86. Tan C, Wang J, Sun B. Biopolymer-liposome hybrid systems for controlled delivery of bioactive compounds: recent advances. *Biotechnol Adv* 2021;**48**:107727.
87. Danhier F, Feron O, Preat V. To exploit the tumor microenvironment: passive and active tumor targeting of nanocarriers for anti-cancer drug delivery. *J Control Release* 2010;**148**:135−46.
88. Tsoi KM, MacParland SA, Ma XZ, Spetzler VN, Echeverri J, Ouyang B, et al. Mechanism of hard-nanomaterial clearance by the liver. *Nat Mater* 2016;**15**:1212−21.
89. Yip BH. Recent advances in CRISPR/Cas9 delivery strategies. *Biomolecules* 2020;**10**:839.
90. Elliott RO, He M. Unlocking the power of exosomes for crossing biological barriers in drug delivery. *Pharmaceutics* 2021;**13**:122.
91. Yim N, Ryu SW, Choi K, Lee KR, Lee S, Choi H, et al. Exosome engineering for efficient intracellular delivery of soluble proteins using optically reversible protein-protein interaction module. *Nat Commun* 2016;**7**:12277.
92. Yang T, Martin P, Fogarty B, Brown A, Schurman K, Phipps R, et al. Exosome delivered anticancer drugs across the blood-brain barrier for brain cancer therapy in danio rerio. *Pharm Res (N Y)* 2015;**32**:2003−14.
93. Luan X, Sansanaphongpricha K, Myers I, Chen H, Yuan H, Sun D. Engineering exosomes as refined biological nanoplatforms for drug delivery. *Acta Pharmacol Sin* 2017;**38**:754−63.
94. Kim G, Lee Y, Ha J, Han S, Lee M. Engineering exosomes for pulmonary delivery of peptides and drugs to inflammatory lung cells by inhalation. *J Control Release* 2021;**330**:684−95.
95. Song H, Liu B, Dong B, Xu J, Zhou H, Na S, et al. Exosome-based delivery of natural products in cancer therapy. *Front Cell Dev Biol* 2021;**9**:650426.
96. Oskouie MN, Aghili Moghaddam NS, Butler AE, Zamani P, Sahebkar A. Therapeutic use of curcumin-encapsulated and curcumin-primed exosomes. *J Cell Physiol* 2019;**234**:8182−91.
97. Munagala R, Aqil F, Jeyabalan J, Gupta RC. Bovine milk-derived exosomes for drug delivery. *Cancer Lett* 2016;**371**:48−61.
98. Agrawal AK, Aqil F, Jeyabalan J, Spencer WA, Beck J, Gachuki BW, et al. Milk-derived exosomes for oral delivery of paclitaxel. *Nanomedicine* 2017;**13**:1627−36.
99. Liang G, Zhu Y, Ali DJ, Tian T, Xu H, Si K, et al. Engineered exosomes for targeted co-delivery of miR-21 inhibitor and chemotherapeutics to reverse drug resistance in colon cancer. *J Nanobiotechnol* 2020;**18**:10.
100. Wu JY, Li YJ, Hu XB, Huang S, Xiang DX. Preservation of small extracellular vesicles for functional analysis and therapeutic applications: a comparative evaluation of storage conditions. *Drug Deliv* 2021;**28**:162−70.
101. Zhang Y, Bi J, Huang J, Tang Y, Du S, Li P. Exosome: a review of its classification, isolation techniques, storage, diagnostic and targeted therapy applications. *Int J Nanomed* 2020;**15**:6917−34.
102. Nikfarjam S, Rezaie J, Kashanchi F, Jafari R. Dexosomes as a cell-free vaccine for cancer immunotherapy. *J Exp Clin Cancer Res* 2020;**39**:258.
103. Naseri M, Bozorgmehr M, Zöller M, Ranaei Pirmardan E, Madjd Z. Tumor-derived exosomes: the next generation of promising cell-free vaccines in cancer immunotherapy. *OncoImmunology* 2020;**9**:1779991.
104. Palucka K, Banchereau J. Dendritic-cell-based therapeutic cancer vaccines. *Immunity* 2013;**39**:38−48.
105. Chiang C, Coukos G, Kandalaft L. Whole tumor antigen vaccines: where are we?. *Vaccines* 2015;**3**:344−72.
106. Batista-Duharte A, Lindblad EB, Oviedo-Orta E. Progress in understanding adjuvant immunotoxicity mechanisms. *Toxicol Lett* 2011;**203**:97−105.
107. Morishita M, Takahashi Y, Matsumoto A, Nishikawa M, Takakura Y. Exosome-based tumor antigens−adjuvant co-delivery utilizing genetically engineered tumor cell-derived exosomes with immunostimulatory CpG DNA. *Biomaterials* 2016;**111**:55−65.
108. Zhang Y, Fang Z, Li R, Huang X, Liu Q. Design of outer membrane vesicles as cancer vaccines: a new toolkit for cancer therapy. *Cancers* 2019;**11**:1314.
109. Li M, Zhou H, Yang C, Wu Y, Zhou X, Liu H, et al. Bacterial outer membrane vesicles as a platform for biomedical applications: an update. *J Control Release* 2020;**323**:253−68.

110. Huang W, Shu C, Hua L, Zhao Y, Xie H, Qi J, et al. Modified bacterial outer membrane vesicles induce autoantibodies for tumor therapy. *Acta Biomater* 2020;**108**:300−12.
111. Cheng K, Zhao R, Li Y, Qi Y, Wang Y, Zhang Y, et al. Bioengineered bacteria-derived outer membrane vesicles as a versatile antigen display platform for tumor vaccination *via* plug-and-display technology. *Nat Commun* 2021;**12**:2041.
112. Schreurs MW, Eggert AA, de Boer AJ, Vissers JL, van Hall T, Offringa R, et al. Dendritic cells break tolerance and induce protective immunity against a melanocyte differentiation antigen in an autologous melanoma model. *Cancer Res* 2000;**60**:6995−7001.
113. Baker SM, Settles EW, Davitt C, Gellings P, Kikendall N, Hoffmann J, et al. *Burkholderia pseudomallei* OMVS derived from infection mimicking conditions elicit similar protection to a live-attenuated vaccine. *NPJ Vaccines* 2021;**6**:18.
114. Pinky, Gupta S, Krishnakumar V, Sharma Y, Dinda AK, Mohanty S. Mesenchymal stem cell derived exosomes: a nano platform for therapeutics and drug delivery in combating COVID-19. *Stem Cell Rev Rep* 2021;**17**:33−43.
115. Drurey C, Coakley G, Maizels RM. Extracellular vesicles: new targets for vaccines against helminth parasites. *Int J Parasitol* 2020;**50**: 623−33.
116. Avila-Calderón ED, Ruiz-Palma MDS, Aguilera-Arreola MG, Velázquez-Guadarrama N, Ruiz EA, Gomez-Lunar Z, et al. Outer membrane vesicles of Gram-negative bacteria: an outlook on biogenesis. *Front Microbiol* 2021;**12**:557902.
117. Shockman GD, Barrett JF. Structure, function, and assembly of cell walls of Gram-positive bacteria. *Annu Rev Microbiol* 1983;**37**: 501−27.
118. Brown L, Wolf JM, Prados-Rosales R, Casadevall A. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nat Rev Microbiol* 2015;**13**:620−30.
119. Gill S, Catchpole R, Forterre P. Extracellular membrane vesicles in the three domains of life and beyond. *FEMS Microbiol Rev* 2019;**43**: 273−303.
120. Schwechheimer C, Kuehn MJ. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat Rev Microbiol* 2015; **13**:605−19.
121. Micoli F, MacLennan CA. Outer membrane vesicle vaccines. *Semin Immunol* 2020;**50**:101433.
122. Raeven RH, van der Maas L, Tilstra W, Uittenbogaard JP, Bindels TH, Kuipers B, et al. Immunoproteomic profiling of bordetella pertussis outer membrane vesicle vaccine reveals broad and balanced humoral immunogenicity. *J Proteome Res* 2015;**14**: 2929−42.
123. Gerritzen MJH, Martens DE, Wijffels RH, van der Pol L, Stork M. Bioengineering bacterial outer membrane vesicles as vaccine platform. *Biotechnol Adv* 2017;**35**:565−74.
124. Fantappiè L, de Santis M, Chiarot E, Carboni F, Bensi G, Jousson O, et al. Antibody-mediated immunity induced by engineered escherichia coli omvs carrying heterologous antigens in their lumen. *J Extracell Vesicles* 2014;**3**:24015.
125. Watkins HC, Rappazzo CG, Higgins JS, Sun X, Brock N, Chau A, et al. Safe recombinant outer membrane vesicles that display M2e elicit heterologous influenza protection. *Mol Ther* 2017;**25**: 989−1002.
126. Frasch CE. Preparation of bacterial polysaccharide-protein conjugates: analytical and manufacturing challenges. *Vaccine* 2009;**27**: 6468−70.
127. Maira-Litran T, Kropec A, Goldmann DA, Pier GB. Comparative opsonic and protective activities of *Staphylococcus aureus* conjugate vaccines containing native or deacetylated staphylococcal poly-*n*-acetyl-beta-(1-6)-glucosamine. *Infect Immun* 2005;**73**: 6752−62.
128. Skurnik D, Cywes-Bentley C, Pier GB. The exceptionally broad-based potential of active and passive vaccination targeting the conserved microbial surface polysaccharide pnag. *Expert Rev Vaccines* 2016;**15**:1041−53.
129. Stevenson TC, Cywes-Bentley C, Moeller TD, Weyant KB, Putnam D, Chang YF, et al. Immunization with outer membrane vesicles displaying conserved surface polysaccharide antigen elicits broadly antimicrobial antibodies. *Proc Natl Acad Sci U S A* 2018; **115**:E3106−15.
130. Holst J, Martin D, Arnold R, Huergo CC, Oster P, O'Hallahan J, et al. Properties and clinical performance of vaccines containing outer membrane vesicles from neisseria meningitidis. *Vaccine* 2009; **27**(Suppl 2):B3−12.
131. Claassen I, Meylis J, van der Ley P, Peeters C, Brons H, Robert J, et al. Production, characterization and control of a neisseria meningitidis hexavalent class 1 outer membrane protein containing vesicle vaccine. *Vaccine* 1996;**14**:1001−8.
132. Balhuizen MD, Veldhuizen EJA, Haagsman HP. Outer membrane vesicle induction and isolation for vaccine development. *Front Microbiol* 2021;**12**:629090.
133. Martins P, Machado D, Theizen TH, Guarnieri JPO, Bernardes BG, Gomide GP, et al. Outer membrane vesicles from neisseria meningitidis (proteossome) used for nanostructured Zika virus vaccine production. *Sci Rep* 2018;**8**:8290.
134. Shehata MM, Mostafa A, Teubner L, Mahmoud SH, Kandeil A, Elshesheny R, et al. Bacterial outer membrane vesicles (OMVs)-based dual vaccine for influenza a H1N1 virus and mers-cov. *Vaccines (Basel)* 2019;**7**:46.
135. Wise J. COVID-19: delta variant doubles risk of hospital admission compared with alpha variant, study shows. *BMJ* 2021;**374**:n2152.
136. van Riel D, de Wit E. Next-generation vaccine platforms for COVID-19. *Nat Mater* 2020;**19**:810−2.
137. Park KS, Sun X, Aikins ME, Moon JJ. Non-viral COVID-19 vaccine delivery systems. *Adv Drug Deliv Rev* 2021;**169**:137−51.
138. Gan Y, Li C, Peng X, Wu S, Li Y, Tan JPK, et al. Fight bacteria with bacteria: bacterial membrane vesicles as vaccines and delivery nanocarriers against bacterial infections. *Nanomedicine* 2021;**35**: 102398.
139. Samsonraj RM, Raghunath M, Nurcombe V, Hui JH, van Wijnen AJ, Cool SM. Concise review: multifaceted characterization of human mesenchymal stem cells for use in regenerative medicine. *Stem Cells Transl Med* 2017;**6**:2173−85.
140. Zhao T, Sun F, Liu J, Ding T, She J, Mao F, et al. Emerging role of mesenchymal stem cell-derived exosomes in regenerative medicine. *Curr Stem Cell Res Ther* 2019;**14**:482−94.
141. Zhao AG, Shah K, Cromer B, Sumer H. Mesenchymal stem cell-derived extracellular vesicles and their therapeutic potential. *Stem Cell Int* 2020;**2020**:8825771.
142. Yeo RW, Lai RC, Zhang B, Tan SS, Yin Y, Teh BJ, et al. Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery. *Adv Drug Deliv Rev* 2013;**65**:336−41.
143. Wang M, Wang C, Chen M, Xi Y, Cheng W, Mao C, et al. Efficient angiogenesis-based diabetic wound healing/skin reconstruction through bioactive antibacterial adhesive ultraviolet shielding nanodressing with exosome release. *ACS Nano* 2019;**13**:10279−93.
144. Fan J, Lee CS, Kim S, Chen C, Aghaloo T, Lee M. Generation of small RNA-modulated exosome mimetics for bone regeneration. *ACS Nano* 2020;**14**:11973−84.
145. Hade MD, Suire CN, Suo Z. Mesenchymal stem cell-derived exosomes: applications in regenerative medicine. *Cells* 2021;**10**:1959.
146. Yao J, Huang K, Zhu D, Chen T, Jiang Y, Zhang J, et al. A minimally invasive exosome spray repairs heart after myocardial infarction. *ACS Nano* 2021;**15**:11099−111.
147. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet* 2020;**395**: 1054−62.
148. Sengupta V, Sengupta S, Lazo A, Woods P, Nolan A, Bremer N. Exosomes derived from bone marrow mesenchymal stem cells as treatment for severe COVID-19. *Stem Cell Dev* 2020;**29**:747−54.
149. Khalaj K, Figueira RL, Antounians L, Lauriti G, Zani A. Systematic review of extracellular vesicle-based treatments for lung injury: are

EVs a potential therapy for COVID-19?. *J Extracell Vesicles* 2020;**9**:1795365.
150. Huang J, Xiong J, Yang L, Zhang J, Sun S, Liang Y. Cell-free exosome-laden scaffolds for tissue repair. *Nanoscale* 2021;**13**:8740−50.
151. Su N, Hao Y, Wang F, Hou W, Chen H, Luo Y. Mesenchymal stromal exosome-functionalized scaffolds induce innate and adaptive immunomodulatory responses toward tissue repair. *Sci Adv* 2021;**7**:eabf7207.
152. Escors D, Breckpot K. Lentiviral vectors in gene therapy: their current status and future potential. *Arch Immunol Ther Exp* 2010;**58**:107−19.
153. Rabinowitz J, Chan YK, Samulski RJ. Adeno-associated virus (AAV) *versus* immune response. *Viruses* 2019;**11**:102.
154. Duan L, Xu L, Xu X, Qin Z, Zhou X, Xiao Y, et al. Exosome-mediated delivery of gene vectors for gene therapy. *Nanoscale* 2021;**13**:1387−97.
155. Zhan T, Rindtorff N, Betge J, Ebert MP, Boutros M. CRISPR/Cas9 for cancer research and therapy. *Semin Cancer Biol* 2019;**55**:106−19.
156. Kim EJ, Kang KH, Ju JH. CRISPR-Cas9: a promising tool for gene editing on induced pluripotent stem cells. *Korean J Intern Med* 2017;**32**:42−61.
157. Kim SM, Yang Y, Oh SJ, Hong Y, Seo M, Jang M. Cancer-derived exosomes as a delivery platform of CRISPR/Cas9 confer cancer cell tropism-dependent targeting. *J Control Release* 2017;**266**:8−16.
158. Sato YT, Umezaki K, Sawada S, Mukai SA, Sasaki Y, Harada N, et al. Engineering hybrid exosomes by membrane fusion with liposomes. *Sci Rep* 2016;**6**:21933.
159. Lin Y, Wu J, Gu W, Huang Y, Tong Z, Huang L, et al. Exosome-liposome hybrid nanoparticles deliver CRISPR/Cas9 system in mscs. *Adv Sci (Weinh)* 2018;**5**:1700611.
160. Li Z, Zhou X, Wei M, Gao X, Zhao L, Shi R, et al. *In vitro* and *in vivo* RNA inhibition by CD9-HuR functionalized exosomes encapsulated with miRNA or CRISPR/dCas9. *Nano Lett* 2018;**19**:19−28.
161. Ye Y, Zhang X, Xie F, Xu B, Xie P, Yang T, et al. An engineered exosome for delivering sgRNA: Cas9 ribonucleoprotein complex and genome editing in recipient cells. *Biomater Sci* 2020;**8**:2966−76.
162. Yao X, Lyu P, Yoo K, Yadav MK, Singh R, Atala A, et al. Engineered extracellular vesicles as versatile ribonucleoprotein delivery vehicles for efficient and safe CRISPR genome editing. *J Extracell Vesicles* 2021;**10**:e12076.
163. Lu TX, Rothenberg ME. Microrna. *J Allergy Clin Immunol* 2018;**141**:1202−7.
164. Huang J, Yu M, Yin W, Liang B, Li A, Li J, et al. Development of a novel rnai therapy: engineered mir-31 exosomes promoted the healing of diabetic wounds. *Bioact Mater* 2021;**6**:2841−53.
165. Khani AT, Sharifzad F, Mardpour S, Hassan ZM, Ebrahimi M. Tumor extracellular vesicles loaded with exogenous Let-7i and miR-142 can modulate both immune response and tumor microenvironment to initiate a powerful anti-tumor response. *Cancer Lett* 2021;**501**:200−9.
166. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* 2011;**29**:341−5.
167. He LF, Xu HW, Chen M, Xian ZR, Wen XF, Chen MN, et al. Activated-PAK4 predicts worse prognosis in breast cancer and promotes tumorigenesis through activation of PI3K/AKT signaling. *Oncotarget* 2017;**8**:17573−85.
168. Thillai K, Sarker D, Wells C. PAK4 pathway as a potential therapeutic target in pancreatic cancer. *Future Oncol* 2018;**14**:579−82.
169. Xu L, Faruqu FN, Lim YM, Lim KY, Liam-Or R, Walters AA, et al. Exosome-mediated RNAi of PAK4 prolongs survival of pancreatic cancer mouse model after loco-regional treatment. *Biomaterials* 2021;**264**:120369.
170. Li Z, Zhao P, Zhang Y, Wang J, Wang C, Liu Y, et al. Exosome-based Ldlr gene therapy for familial hypercholesterolemia in a mouse model. *Theranostics* 2021;**11**:2953−65.
171. Orefice NS. Development of new strategies using extracellular vesicles loaded with exogenous nucleic acid. *Pharmaceutics* 2020;**12**:705.
172. Lamichhane TN, Raiker RS, Jay SM. Exogenous DNA loading into extracellular vesicles via electroporation is size-dependent and enables limited gene delivery. *Mol Pharm* 2015;**12**:3650−7.
173. Haney MJ, Klyachko NL, Zhao Y, Gupta R, Plotnikova EG, He Z, et al. Exosomes as drug delivery vehicles for Parkinson's disease therapy. *J Control Release* 2015;**207**:18−30.
174. Podolak I, Galanty A, Sobolewska D. Saponins as cytotoxic agents: a review. *Phytochemistry Rev* 2010;**9**:425−74.
175. Wu JY, Li YJ, Hu XB, Huang S, Luo S, Tang T, et al. Exosomes and biomimetic nanovesicles-mediated anti-glioblastoma therapy: a head-to-head comparison. *J Control Release* 2021;**336**:510−21.