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Extracellular vesicles: A comprehensive review of classification, isolation, characterization, and cargo loading

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ABSTRACT: Extracellular vesicles (EVs) are submicron membrane-bound structures released from various cell types into extracellular space. EVs are divided by biogenesis into exosomes, microvesicles and apoptotic bodies; moreover, they can also be subtyped into natural, engineered and hybrid EVs. EVs play a vital role in cell-to-cell communication, allowing cells to exchange cargos including proteins, lipids and nucleic acid materials, therefore making them valuable tools as novel biomarkers of diseases, as therapeutic agents and as drug delivery messengers. In this review, we describe several methods for isolation and characterization of EVs. Furthermore, engineered EVs as target drug delivery systems as well as recent advances in hybrid EVs and the engineering of EVs with synthetic lipid nanoparticles will also be discussed.

KEYWORDS: extracellular vesicles, engineered EVs, hybrid EVs, cell-to-cell communication, drug delivery vehicles, therapeutic agents

INTRODUCTION

Extracellular vesicles (EVs) are nano-sized lipid bilayer vesicles released from almost all prokaryotic and eukaryotic cells under both normal and pathological conditions. EVs are generally classified into three main subtypes depending on their size and modes of biogenesis. Exosomes are 30–150 nm membrane-bound vesicles with cup-shaped morphology and are formed by inward budding of endosomal membranes into the lumen and progressively form intraluminal vesicles (ILVs) within large multivesicular bodies (MVBs). After fusion of MVBs with plasma membrane, ILVs are expelled into extracellular milieu in the form of exosomes. Ectosomes (microvesicles or microparticles) are heterogeneous membrane vesicles of 100–1000 nm in size and are released by outward budding directly from plasma membrane in response to activation. Apoptotic bodies, a final subtype of EVs, are formed through a process termed programmed cell death characterized by a series of regulated morphological steps, including DNA fragmentation, membrane protrusion formation, and plasma membrane blebbing with the size of 800–5000 nm in diameter [1]. In addition, various terms have been used to define EV subtypes. For example, small EVs refer to those EVs smaller than 200 nm, while large EVs refer to those larger than 200 nm, as classified based on their size. Oncosomes describe EVs derived from cancer cells and cardiosomes refer to EVs involved in cardiac signaling and repair during myocardial infarction and ischemic injury [2, 3].

It has been shown that physiological and pathological conditions affect EV number and composition. These findings lead to exploring EVs as diagnostic and prognostic biomarkers in many diseases [1, 4, 5]. EVs can also act as a cargo carrier capable of transporting exogenous biomolecules, such as microRNAs (miRNAs), proteins and lipids, from donor cells to recipient cells. This intercellular communication highlights the crucial roles of EVs in health and diseases. Additionally, EVs are excellent therapeutic vehicle due to their higher biocompatibility and less toxicity when compared to synthetic drugs. They are also non-immunogenic and capable of diffusing into the blood, penetrating into tissues, targeting various cells, and even crossing the blood-brain barrier [5–10]. Furthermore, EVs are also highly engineerable [11, 12]. Engineering of EVs by packaging certain chemical drugs or biomolecules as cell-free drug delivery vehicles has a great potential for precise targeting [13, 14].

In this review, the isolation and characterization of EVs according to Minimal Information for Studies of Extracellular Vesicles 2023 (MISEV2023) guidelines [15] is described. An overview of the current knowledge of EVs and approaches to studying EVs are pro-

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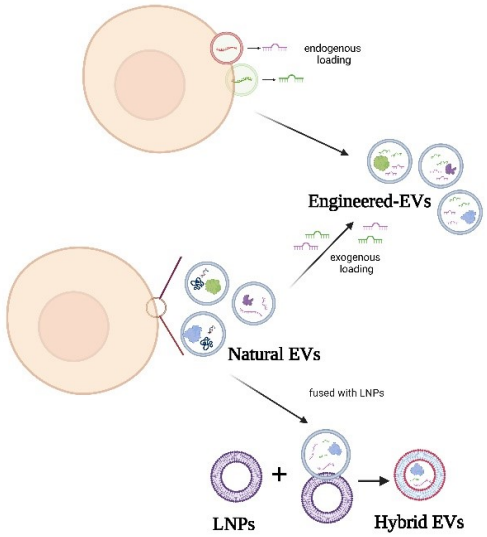

Fig. 1 Type of EVs: natural EVs, engineered EVs and hybrid EVs (Created with https://BioRender.com/z81x007).

vided. We also cover different engineered EVs, combining targeted cell-type specific EVs and biomolecule encapsulation for therapeutic applications. Recent advancements in hybrid EVs and the engineering of EVs with synthetic lipid nanoparticles (LNPs), particularly focusing on strategies for various targeting and therapeutic applications, will also be discussed.

TYPE OF EXTRACELLULAR VESICLES

According to the recent MISEV2023 guidelines, the terminology of EVs is defined as particles that are released from cells, enclosed by a lipid bilayer, and incapable of self-replication [15]. Generally, EVs are classified based on their size, biogenesis, function, modification, and applications; here we will focus on the classification of EVs into natural, engineered, and hybrid EVs (Fig. 1).

Natural EVs

Natural EVs are released from the cells in physiological, pathological, and activator-induced conditions. Each cell type produces EVs with unique biomolecule cargo. Natural EVs come from various sources, including blood, cell culture condition medium, urine, and cow's milk. Serum and plasma are common biological fluids used in EV studies as they contain large amounts of EVs from many cell sources. However, these biological fluids also contain lipids and non-vesicular proteins, which might interfere with EV purity. The cell culture supernatant is used to study EVs from a single-cell source. The typical biomedical applications of natural EVs include the study of cellular communication, their role in disease pathogenesis, and their use as biomarkers for diagnosis and prognosis, for example, in cancers, cardiovascular diseases, and neurologic diseases [16–19].

Engineered EVs

Engineered EVs become potential tools for therapeutic delivery systems capable of carrying drugs, nucleic acid, and proteins to specific target cells. These EVs are engineered to modify their surface ligands or internal cargo to enhance their capacity and functionality when compared to natural EVs, such as increasing therapeutic activity by improving the quality and quantity of biomolecule loading or increasing the specificity to target cells and EV's stability. There are two main approaches for engineered EV production. Firstly, endogenous loading of cargos involves enriching the expression of the molecules of interest in the EV-originated cells, then allowing natural EVs production to occur with the enrichment of desired biomolecules [20, 21]. Secondly, exogenous loading of cargos entails incorporating molecules into post-isolated natural EVs [22, 23]. Engineered EVs have been used to deliver biomolecules for treatment in a wide variety of disease models; for example, various types of cancers [20, 24], stroke [25], and atrial fibrillation [26].

Hybrid EVs

Hybrid EVs represent a cutting-edge technology of EV production that combine natural EVs with other nanomaterials such as liposomes, iron nanoparticles, and organic or inorganic nanoparticles [27–29]. These EV types combine the advantages of natural EVs and the advantages of synthetic nanoparticles. Thus, hybrid EVs enhance their natural properties or convey new functionalities that are not present in purely natural or synthetic EVs. For decades, nanoparticle systems have been recognized as breakthroughs for efficient drug delivery. The suitable nanoparticle transports its drug payload effectively to a specific target, where it can release the drug either inside the cell or in the extracellular environment, allowing for direct uptake and therapeutic action. During transportation, the nanoparticle prevents undesirable interactions with non-target tissues, prolongs the circulation time of the encapsulated drug, and supports controlled release.

The concept of integrating natural EVs with nanoparticles (NPs), such as polymers or liposomes, has led to the creation of what are termed hybrid EVs. This has emerged as an alternative approach and a promising strategy in drug delivery systems (DDS). These hybrid systems address several limitations associated with natural EVs, including cargo loading, circulation stability, and targeting capabilities [30–32]. This review explores the preparation methods, types of liposomes used, and applications of hybrid EVs, with a focus on their potential in cancer and cardiovascular disease (CVD) therapies.

ISOLATION OF EXTRACELLULAR VESICLES

The standard methods for EV isolation are based on four main principles: centrifugation, filtration, chromatography, and precipitation. (i) The centrifugation-based methods are conventional methods for EV isolation. Differential ultracentrifugation (UC) and density gradient ultracentrifugation (DG-UC), such as cushion or iodixanol and sucrose gradient centrifugation, separate EVs based on size and density. Although they are proposed as the gold standard method for EV isolation, they can be time-consuming, require large amounts of samples and expensive equipment, and have low throughput and limited scalability. (ii) The filtration-based methods, such as ultrafiltration and tangential flow filtration (TFF), separate EVs based on their size by using porous membranes with specific membrane types and pore sizes to trap EVs of particular size. However, these methods are at risk of membrane clogging by accumulated particles. Clogging not only prolongs the isolation process but can also affect the purity and yield of the recovered EVs. (iii) The chromatography-based methods include size exclusion chromatography (separation based on molecular size), anion exchange chromatography (separation based on net charge differences), and immunoaffinity chromatography (separation based on specific binding between surface marker proteins of EVs and immobilized antibodies coated column). These methods enhance specificity and enrich target EV populations but are limited in quantities of samples processed and the amount of recovered EVs. (iv) The precipitation-based method, such as polyethylene glycol (PEG) polymer-based precipitation, separates EVs based on the aggregation of EVs in the precipitated solution. This approach is a cost-effective and scalable method but requires prolonged incubation and additional processes to remove the polymer from isolated EVs. Since 2020, the field of EV isolation has been invigorated by the development of new technologies. These innovative methods, detailed in Table 1, hold great potential for the future of EV isolation.

The selection of suitable EV isolation methods depends on many factors, such as the type of EV source, the quantity and quality of source material, the specific type of isolated EVs, the purity and yield of isolated EVs, available instruments, and downstream applications. The efficiency of each method is evaluated by purity, yield, morphology of enriched EVs, time efficiency, and the requirement for specialized instruments. Combining these methods provides significant benefits with high purity of isolated EVs, but the recovery amount might decrease. Many studies compared these methods in terms of purity, yield, and characteristics of isolated EVs, their biomolecule content, and their downstream analysis compatibility [33–37].

CHARACTERIZATION OF EXTRACELLULAR VESICLES

EVs characterization is the critical step following their separation to achieve downstream application in the diagnosis or therapeutic. Several approaches are employed to confirm isolated EVs' physical and functional properties. According to the MISEV2023 recommendations, characterization of EVs should include quantification in terms of particle number concentration and particle size, EV morphology, and detection of specific EV and non-EV protein markers. In addition, the quantification of total protein, total lipids, and total RNA in isolated EV samples, as well as the localization of EV-associated components are recommended [15].

The morphology of EVs can be analyzed by a diverse range of electron microscopy approaches, such as transmission electron microscopy, scanning electron microscopy, and cryogenic electron microscopy, each offering unique insights [48]. The concentration of EVs is generally determined by nanoparticle tracking analysis (NTA), which reports the number of EV particles versus particle size; and dynamic light scattering (DLS), which efficiently analyzes the overall number of EVs in solution [49, 50]. NTA is appropriate for polydisperse samples such as EVs from biological fluids, while DLS is suitable for rapid analysis of monodisperse samples such as EVs from culture supernatant. Other methods include resistive pulse sensing (RPS) which measures the concentration and diameter of particles along with their zeta potential, and single-particle interferometric reflectance imaging sensing (SP-IRIS) which measures the size, concentration, and identifies EV surface markers [51, 52]. The zeta potential measurement at the EV surfaces is useful for analyzing surface charge changes after EV modification and assessing stability [53]. Western blotting and flow cytometry are widely used for evaluating EV markers, their cellular origin, and protein cargo. Western blotting requires large amounts of EV proteins and the lysis of EVs; therefore, it cannot differentiate between surface-expressed and intravesicular proteins. Conventional flow cytometers have a detection limit of 300–500 nm for EVs, whereas high-resolution flow cytometers (hFCM) with high-sensitivity laser scattering are suitable for the analysis of single small EVs. For functional tracking and localization of EVs in target cells, fluorescence microscopy techniques such as diffraction-limited fluorescence microscopy, total internal reflection microscopy (TIRF-M), confocal microscopy, and light-sheet microscopy are used. In addition, imaging flow cytometry is used for studies of EV internalization [15, 54].

ENGINEERED EXTRACELLULAR VESICLES

Methods of loading cargo

Recently, engineered EVs are mostly produced from stem cells (especially mesenchymal stem

Table 1 Recent technologies for EV isolation.

Method	Principle of isolation	Type of sample	Advantages	Limitations
Asymmetrical flow field-flow fractionation (AF4) [38]	Field flow fractionation	Plasma and serum	High purity, scalable	Limited by sample viscosity and concentration, resolution limits for EVs with very similar hydrodynamic sizes
Electric field assisted tangential flow filtration system (E-TFF) [39]	Size-based filtration with electrophoretic migration-based separation	Cell-culture conditioned media	Improve purity, increase yield, and reduce isolation time compared to UC	Membranes can be damaged by strong electric field
Column-based CD9-anti body-immobilized HPLC immunoaffinity chromatography [40]	Immunoaffinity chromatography	Serum	Enhance specificity and enrich EVs at microliter scale	Potential loss of EV subpopulations that have no or low CD9 expression
Paramagnetic bead-based surface epitope immunoaffinity [41]	Immunoaffinity-based isolation	Plasma	High-throughput, improve specificity in isolating EV subpopulations	Unable to release and collect intact EVs from magnetic bead
Bifunctional immunoaffinity magnetic nanoparticles [42]	Immunoaffinity-based isolation under a magnetic field	Cell-culture conditioned media	High purity enrichment performance	Potential loss of EV subpopulations that have no or low expression of selected marker
Polysaccharide chitosan-based magnetic beads [43]	Affinity-based isolation under a magnetic field	Cell-culture conditioned media, saliva, urine, plasma, and serum	Simple, high-throughput, applicable to various biological fluids	Limited specificity, Lower purity with non-EV contaminants
Gold-nanoparticle-coated silicon (Si) wafer [44]	Immunoaffinity-based isolation	Serum	Simple and reusable Si wafer, compatible with microfluidic platforms	Potential loss of EV subpopulations that have no or low expression of selected marker
Exosome precipitation by ionic strength modulation: ExoPRISM [45]	Precipitation	Culture medium, urine, plasma, and serum	Simple, low cost, user-friendly, readily scalable, applicable to a broad range of biological fluids	Low purity
NTI-EXO precipitation [46]	Precipitation	Serum and plasma	More efficient for smaller sample sizes	Low purity and potential non-EV contaminant
Immuno-magnetophoresis-based microfluidic chip [47]	Microfluidic platform	Conditioned media	Integrated multiple functions: isolation and detection, rapid preparation, require small sample volumes	Magnetic beads or antibodies may carry over and interfere with downstream assay

cells) [20, 25, 26] or cancer cell lines (especially HEK293T/HEK293FT cells) [20–22, 24]. The modification of EV-originated cells can occur through viral vector transduction or transient transfection with recombinant plasmids expressing miRNAs, siRNAs, or mRNAs of interest to produce EVs enriched with specific nucleic acids or proteins [20, 21, 24, 55–57].

There are additional methods to enrich the cargo of interest more specifically. For example, fusing target proteins with transmembrane proteins involved in EV biogenesis, particularly CD63, or other proteins that have been demonstrated to be parts of EV, such as PTGFRN and BASP1, has been shown to enhance their inclusion in EVs [57, 58]. However, this approach may not be generalized to all proteins involved in EV biogenesis, potentially due to interference from the fusion proteins [57]. Additionally, when the desired cargo is RNA, EV-enriched proteins fused with an RNA binding domain have been shown to increase RNAs loaded into EVs [56, 58]. Another strategy involves anchoring target proteins to the plasma membrane to enhance their inclusion in EVs. One study achieved this by fusing the target protein with Cryptochrome 2, which could dimerize with its ligand CIBN. Modification of CIBN was performed by adding a short peptide tag known to be myristoylated and palmitoylated and, thus, be anchored to the plasma membrane [21].

Forced expression of the spike glycoprotein of the vesicular stomatitis virus (VSV-G) could induce host cells to increase EV budding from the plasma membrane [55]. The characteristics of these VSV-G induced EVs, as they termed it “gesicles”, do not match those of exosomes and are believed not to originate from the multivesicular bodies [55]. However, they have the advantage in inducing EV release without mechanical or chemical perturbations. Studies have shown that

these EVs are quite stable after freeze/thaw cycles and can be used to deliver biomolecules of interest, including proteins and nucleic acids [55, 59, 60].

Similar to endogenous EVs, exogenous engineered EVs can be produced to transport not only proteins and nucleic acids, but they can also be loaded with other molecules such as drugs as their cargos. Biomolecule loading and surface functionalization can be achieved using techniques such as co-incubation, electroporation, extrusion, freeze-thawing, and sonication [22, 23, 25, 61]. These methods have the advantage that they do not require genetic manipulation of the source cells of EVs.

The selection between endogenous or exogenous loading of cargos to produce engineered EVs depends on several considerations. If the desired EV cargos are gene products, including proteins or miRNAs, it is possible to do either transient or stable expression of the desired genes in the originated cells to endogenously load them. Stable expression would allow multiple rounds of EV production without having to genetically manipulate the originated cells every time [20]. However, for drugs that cells cannot produce, they would need to be supplemented into the originated cells or in the post-released EVs in order to enrich them [62, 63]. And for biomolecules that are toxic to the originated cells, it might be difficult to use endogenous loading techniques to highly enrich them in EVs because the viability of originated cells may decrease. On the other hand, there are also limitations in the amount of cargo that could be incorporated into EVs by exogenous loading techniques.

Targeting specific cell types

While natural EVs may have their preferred target cells, in order to use EVs in drug and/or treatment delivery, researchers also investigated methods to make EVs more specific to desired target cells than to normal cells or other cell types. The main method is to incorporate targeting molecules. For example, it has been shown that fusing a membrane protein Lamp2b in EVs to a neuro-specific RVG peptide sequence could target the EVs to the brain [25].

Endosomal escape and reaching target cellular compartments

Even when EVs get to the desired recipient cell types, some studies still encountered the problems of getting the cargo out of the endosomes to the target cellular compartments. There have been several methods to enhance the endosomal escape; for example, VSV-G has been incorporated into the EVs' membrane, though not fused to cargo molecules, to improve membrane fusion and cargo release from the endosomes [21]. Other chemical compounds have also been demonstrated to improve endosomal escape of EV proteins [64]. Then, after EVs manage to release their content from the endosome, methods have also been employed to send them to where they should function. For example, a nuclear localization signal was added at the N-terminus of EV-loaded Cas9 protein to get it to the nucleus to perform DNA cleavage [21].

HYBRID EXTRACELLULAR VESICLES

The preparation methods for hybrid EVs

In general, the methods used to prepare hybrid EVs rely on physicochemical techniques. These involve the mixing of EVs and NPs through electrostatic or hydrophobic interactions, facilitated by the temporary disruption or permeabilization of the lipid membrane, which then reassembles to form a hybrid complex, or through the fusion of lipid layers. A previous study reported that amphiphilic cationic nanogels were successfully combined with EVs through electrostatic and hydrophobic interactions between their cholesteryl groups and the lipid bilayer of EVs. The complex formation improves the stability and enhances its cellular uptake [65]. The hybridization of EVs and poly(lactic-co-glycolic acid) (PLGA) NPs functionalized with aptamers was prepared using microfluidic sonication. By the transient opening of lipid bilayers, core–shell structures could be formed and EVs act as a shell. The resulting hybrid EVs exhibited a prolonged *in vivo* circulation time [66].

Recently, hybrid membrane engineering of EVs joining with various types of LNPs has been reported. Liposomes, a category of synthetic LNPs, are preferred as DDS due to self-assembly characteristics, ability to encapsulate both water-soluble and lipophilic drugs, and improved pharmacokinetic profile [67]. The strategy of hybridizing EVs with liposomes has been employed to improve the surface characteristics of EVs in order to alter their immunogenicity, improve colloidal stability, extend their half-life in circulation, and promote cellular uptake [68]. Due to the similar surface properties of EVs and liposomes which are lipid-based, the hybridization of EVs and liposomes is prepared by fusion of lipid bilayers. Common methods for achieving membrane fusion include incubation, sonication, and freeze-thaw cycles [69].

Several studies have been carried out on EVs/liposome hybrids to assess their potential as DDS. The composition of liposomes, described in the following subsection, plays a key role in hybrid EVs to obtain the desired characteristics for effective DDS.

Type of liposome used in hybrid EVs

Liposomes consist of numerous phospholipid molecules that allow for customization of the lipid composition. Lipids, from both synthetic and natural sources, have a wide range of head groups, chain lengths, and saturation levels. The different charges of lipids depend on the head groups. Cationic lipids provide a positive charge to the liposome surface. Liposomes containing cationic lipids can promote

Table 2 Summary of hybrid EVs based on the type of liposome.

Type of liposome	EVs source	Technique for EVs isolation	Therapeutic agent	Main findings
Zwitterionic (DOPC-based liposomes) Cationic (DOTAP-based liposomes) Anionic (DOPS-based liposomes) [70]	Raw 264.7 macrophages	Differential centrifugation and micro-filtration	–	Lipid composition of hybrid EVs influenced cellular uptake
Cationic (DOTAP-based liposomes) Anionic (PC was substituted for DOTAP) [73]	Adipose-derived mesenchymal stem cells	Ultracentrifugation	Paclitaxel	The fusion ability of EVs with cationic liposome was better than with anionic liposome.
Anionic DOPS-based, DOPG-based, and BMP-based liposomes [75]	HepG2 cell line	Ultracentrifugation and affinity method	–	Fusion activity depended on the ratio of particle number between EVs and anionic liposomes, liposomes diameter, and pH.
Cationic (DOTAP-based liposomes) [76]	B16–F10 cell line	Differential centrifugation and size exclusion filter	PD-L1 trap plasmid	Hybrid EVs demonstrated effective penetration into lymph nodes, promoting dendritic cell maturation and activating cytotoxic T cell.
Cationic (DODEAC-based liposomes) [78]	MCF-7 cell line	Differential centrifugation	siRNA	Hybrid EVs improved efficiency of the siRNA delivery to breast cancer cells.
Cationic (DODAG-based liposomes) [79]	Skimmed bovine milk	Differential ultracentrifugation	siRNA	Hybrid EVs-liposome exhibited higher stability in simulated intestinal fluid compared to liposome.
Ionizable (DLin-MC3-DMA-based liposomes) [80]	Cardiac progenitor cells	Tangential flow filtration (TFF) and size exclusion chromatography (SEC)	siRNA	Hybrid EVs were effective in delivering siRNA to various cell types, with altered cellular uptake, reduced toxicity, and retained gene-silencing effects.

electrostatic interactions with cell membranes and negatively charged DNA. Anionic lipids, which are negatively charged, are more similar to cell membranes and the membranes of EVs. Zwitterionic lipids, the neutral lipids, compose of both positive and negative charges [70–72]. The fusion of EVs with various types of liposomes can generate hybrid EVs exhibiting distinct characteristics (Table 2). The generation of EV-based hybrid systems using cationic liposomes seems to be more widely studied compared to other types of liposomes. Regarding the EV surface, the EV membrane is negatively charged so it can interact with positively charged liposomes when mixed, leading to the fusion of lipid bilayers [73, 74]. A previous report suggested that the cationic liposome had the fusion ability higher than anionic liposome [73]. However, to increase the fusion capacity of anionic liposome, some factors such as the particle number ratio between the EVs and the anionic liposomes, diameter of liposomes, and buffer pH should be considered [75]. The EVs-liposome hybrid systems overcome the limitation of single used EVs by promoting the cellular uptake, stability, and targeting capacity in DDS [70, 73, 76–78]. In addition, the enhancement of immunogenicity using EVs-cationic liposome has been reported, such as in controlling the activation of B cells, production of mediating molecules of the immune response, and lymphocyte-mediated immunity [76]. The choice of liposome type to integrate with EVs depends on the specific objectives of the intended application. Optimizing the stability, targeting capabilities, and functionality of the resulting hybrid EVs can establish them as a highly versatile platform for developing next-generation therapeutic strategies. In addition, to enhance the therapeutic effect, hybrid EVs can be loaded with various types of biomolecules such as siRNA, miRNA, and drugs.

Application of hybrid EVs

The most significant advancements in nanotechnology are focused on the detection and treatment of cancer. Many studies have focused on examining the drug delivery properties of EVs and NPs. These studies have examined a variety of compounds, ranging from large molecules like siRNA, miRNA, and proteins to tiny molecules like paclitaxel (PTX), doxorubicin (DOX), and curcumin [73, 78, 79, 81]. The hybrid EVs, from EVs and liposomes, increase loading efficiency while toxicity remains low. A previous study fused exosomes derived from HEK293FT cells expressing sgRNA with liposomes and loaded with a dCas9-expressing vector by incubating the mixture for 12 h at 37 °C. The resulting hybrids effectively delivered CRISPR–Cas9 to mesenchymal stem cells (MSCs) [82]. Wu et al [83] developed exosome–liposome hybrid NPs using the

freeze-thaw technique. They combined exosomes with ALKBH5 mRNA-loaded liposomes in a 1:1 ratio and subjected the mixture to three cycles of freezing and thawing to facilitate particle fusion. This method effectively addresses the challenge of encapsulating mRNA within exosomes and reduces the toxicity associated with liposomes, significantly enhancing the therapeutic impact on colorectal cancer [83]. The hybridization of exosomes from murine macrophage and liposomes with DOX loaded was shown to enhance pH-sensitive drug release in the acidic tumor microenvironment and increased toxicity toward cancer cells [84]. The hybrid EVs can be engineered to increase the effectiveness at targeting cells and shown to be a great carrier for targeted delivery. Hybrid exosome system by combining folate-targeted liposomes with exosomes of MSCs for delivering the anticancer drug PTX has been reported. The findings indicated that combining EVs with folate-modified liposomes significantly improved target recognition and uptake. Moreover, *in vivo* experiments showed that hybrid EVs significantly inhibited tumor growth in colorectal tumor-bearing mouse models [73].

In addition, recent advancements have explored the development of hybrid nanovesicles through the fusion of liposomes with EVs to improve therapeutic outcomes in CVDs. For example, Tan et al [85] introduced platelet-mimicking hybrid nanovesicles by integrating platelet-derived vesicles with liposomes encapsulating mesoporous silica particles loaded with miR-21. These hybrid EVs demonstrated targeted delivery to monocytes and macrophages in the bloodstream, accumulating in myocardial vascular lesions. The precise delivery of miR-21 facilitated the transition of pro-inflammatory M1 macrophages to anti-inflammatory M2 phenotypes, thereby reducing inflammation and promoting cardiac tissue repair following myocardial ischemia-reperfusion injury. Hybrid EVs by fusing MSC-EVs with monocyte–macrophage membranes were developed by Zhang et al [86] to facilitate the cardiac repair and functional remodeling. *In vitro* and *in vivo* studies showed that integrating monocyte membrane components provided these hybrid NPs with a highly specific targeting ability for injured myocardium by mimicking the recruitment properties of monocytes post-myocardial infarction/reperfusion injury (MI/RI) [86].

The development of hybrid EVs thus represents a significant advancement in DDS, particularly for cancer treatment and cardiovascular therapy. By combining the advantages of natural EVs with the enhanced capabilities of liposomes or other nanoparticles, hybrid systems address key limitations and offer promising potential for effective and targeted drug delivery.

CONCLUSION

Recent advancements in isolation and characterization of EVs allow the study of their properties and the application in DDS. In addition to EVs isolated from natural sources, various methods have also currently been developed to produce engineered EVs and hybrids EVs to further tailor them for specific therapeutic purposes.

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