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REVIEW

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Exploiting the role of milk extracellular vesicles: a comprehensive analysis on isolation methods, characterization, surface modifications, and their therapeutic applications

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Abstract

Milk EVs (MEVs) are a class of extracellular vesicles (EVs) that have attracted significant attention for their potential as natural nanocarriers in drug delivery. These nanosized vesicles (30 - 150 nm) are composed of a lipid bilayer that encapsulates proteins, nucleic acids, and lipids. The biogenesis of EVs involves a tightly regulated "endocytosis-fusion-secretion" pathway, and they are secreted by diverse cells into physiological fluids (blood, urine, and saliva). The isolation and characterization methods of MEVs are essential to achieve high purity, structural integrity, size distribution, and biomolecular composition. Efficient drug loading strategies such as passive diffusion, electroporation, and sonication enable the incorporation of therapeutic molecules. Surface modifications such as PEGylation, ligand conjugation, and genetic engineering further enhance the targeting efficiency, circulation stability, and therapeutic efficacy. Given their biocompatibility, low immunogenicity, and natural ability to traverse biological barriers, MEVs offer a scalable, and non-toxic platform for targeted drug delivery such as span cancer therapy, neurodegenerative disease treatment, and immune modulation. However, further research is needed to optimize MEV-based therapeutics, ensuring their efficacy and safety through rigorous clinical trials. This review explores the biogenesis, composition, isolation, characterization, drug-loading strategies, surface modifications, and therapeutic applications of MEVs, highlighting their emerging role in nanomedicine.

Keywords Milk extracellular vesicles, Isolation, Characterization, Drug loading, Application, Surface modification

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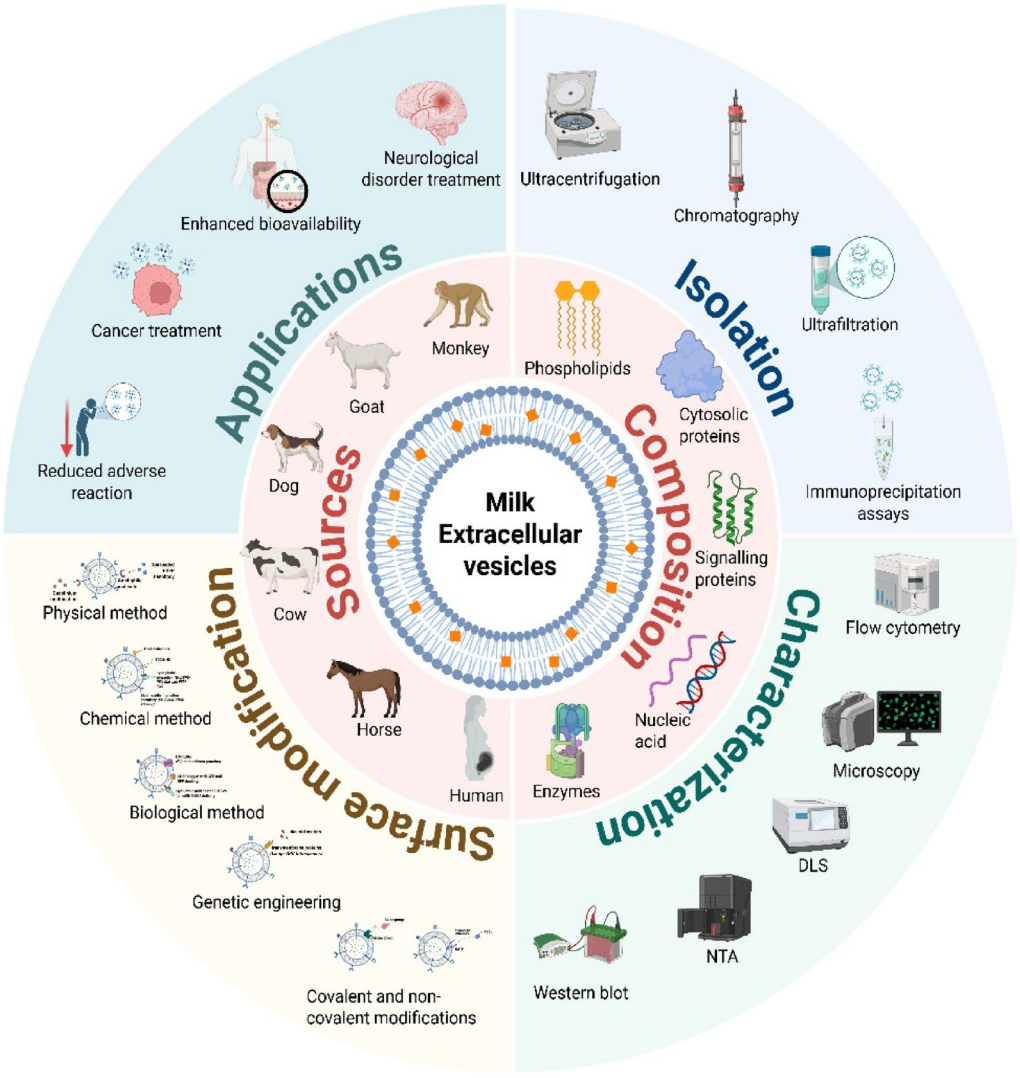
Graphical abstract



Introduction

Historical development of Milk EV research

The conceptual and experimental evolution of Extracellular vesicles (EV) research spans over two decades (Fig. 1). EVs were first discovered in 1983 during sheep reticulocyte differentiation, establishing the foundation of vesicle-mediated research [1]. EVs are spherical nanosized biomaterials that range from 30 to 1000 nm in diameter, are confined by lipid bilayers, and carry diverse biological molecules such as lipids, nucleic acids, and proteins [2]. EVs contain intracellular and membrane proteins, as well as microRNAs and mRNAs, which contribute to their function in intercellular signalling. They facilitate cell-to-cell communication by directly binding EV surface antigens to receptors on target cells and delivering RNAs and proteins to recipient cells. EVs are actively produced by different cells, including those of plants, animals, and malignant origins, and are present in multiple physiological fluids such as ascites, bronchoalveolar lavage fluid, urine, sputum, bile, milk, semen, and amniotic fluid [3, 4]. EVs are being investigated for drug delivery, disease prognosis, and therapeutic uses in disorders, including cancer, neurodegeneration, and cardiovascular diseases, because of their capacity to transport bioactive molecules across physiological barriers [5, 6]. Their ability to focus on certain cell types and tissues makes them attractive candidates for regenerative medical applications [7]. In addition to regulating cell signalling pathways, EVs exhibit anti-inflammatory, antioxidant, and anti-apoptotic properties while also lowering plasma TGF-β levels and influencing IL-6 expression. Early investigations into EV mediated nucleic acid delivery primarily focused on

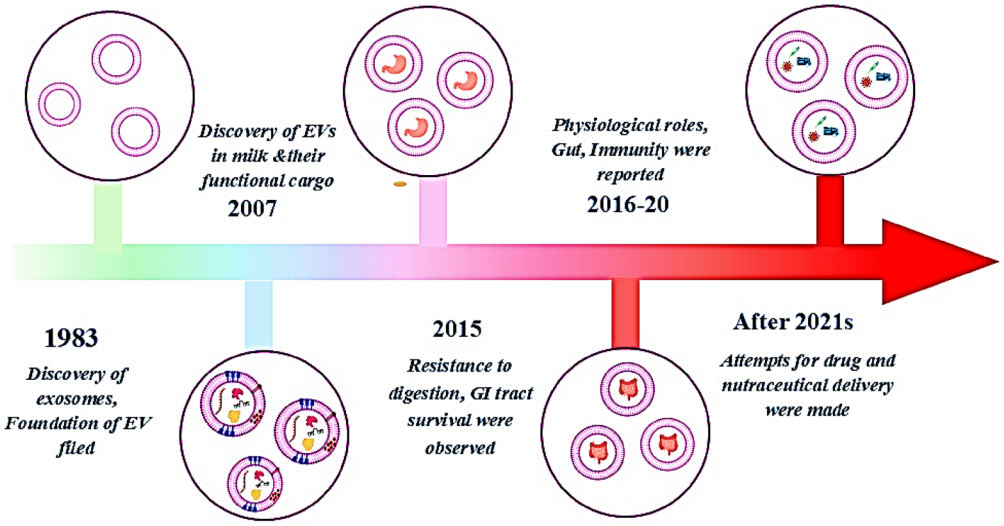


Fig. 1 Timeline of key milestones in MEV research. A chronological representation highlighting major scientific advancements in MEV research. The timeline summarizes foundational discoveries, including the initial identification of vesicle-like structures in microbes, breakthroughs in understanding their biogenesis, and recognition of their roles in intercellular communication

cell culture derived EVs, including mesenchymal stem cell and tumor derived vesicles, whereas MEVs were initially explored mainly for their nutritional and immunological functions rather than as carriers for therapeutic nucleic acid cargo [8]. However, a significant limitation in commercial pharmaceutical manufacturing is the low EV production by cell lines. This difficulty has prompted scientists to investigate more abundant and accessible substitute sources, such as MEVs. The first report describing MEVs appeared in 2007, marking the onset of dairy EV research [9]. Bovine milk is a nutrient-dense diet known to promote growth and development and has significant health benefits because of its immunological properties [10]. Bovine milk is a biocompatible, cost-effective and readily available source of EVs that provide proteins, lipids, vitamins, minerals, and other nutrients essential for immune defence, development, and physiological regulation [22–26]. MEVs have become an intriguing field of study in recent years, garnering attention for their potential roles in nutrition, immune modulation, and intercellular communication [13]. These nanosized membrane-bound structures are naturally found in the milk of various mammalian species such as humans, goats, camels, pigs, and cows [14]. MEVs are composed of a lipid bilayer membrane that contains different bioactive substances, including proteins, lipids, metabolites, and nucleic acids that confers exceptional stability. By 2015, their oral bioavailability and structural stability under simulated gastrointestinal conditions were confirmed, expanding their therapeutic promise [9]. Milk is more economical and accessible than cell culture media and offers additional advantages as a natural oral delivery vesicle, presenting a practical and patient-oriented treatment approach [15]. Between 2016 and 2020, the research landscape rapidly broadened to encompass roles in immunity, gut health, and neural repair. MEVs have been shown to control immunity [16], encourage intestinal epithelial cell growth [17], and act as indicators of cattle diseases [18]. Oral administration of MEVs has shown significant stability against enzymatic degradation and low pH, indicating their potential to endure gastrointestinal transit and deliver cargo to target cells [19]. Their resilience to digestive enzymes, freezing, and thawing is ascribed to their lipid bilayer membrane, which provides protection. The unique composition and biological activities of MEVs (Fig. 2) have sparked interest in their possible applications in food science, pharmaceuticals, biotechnology, and targeted drug delivery systems [20]. 2021 onward, the focus has shifted toward scalable isolation, gene/drug loading strategies, and translational integration into cosmetic and nutraceutical products [21]. Several state-of-the-art biotechnological and pharmaceutical companies are investigating the potential applications of MEVs for the detection and management of medical conditions [22].

Bovine MEVs (BMEVs), nanosized membrane vesicles, penetrate biological barriers [20] and serve as organic carriers, delivering drug and diagnostic agents to diverse organs and tissues [23]. EVs are abundant in bovine milk and their bilayer membranes maintain stability in vitro for extended periods [24]. Bovine MEVs possess advantageous qualities as drug carriers: they are cost-effective, readily available, and stable for large-scale production [25]. Utilizing endocytosis, Tong et al.. demonstrated that BMEVs could effectively cross the gastrointestinal barrier and demonstrate drug-loading efficiencies between

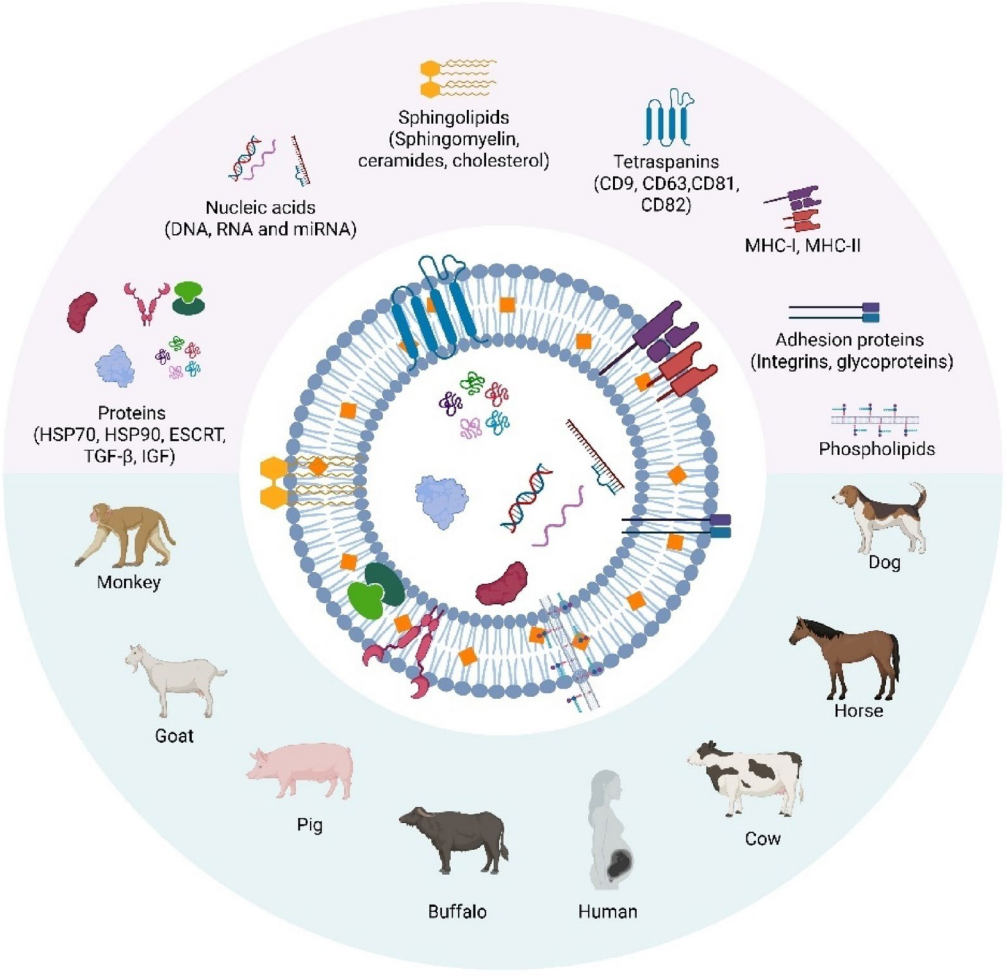


Fig. 2 Composition and sources of MEVs. This figure illustrates the diverse biochemical makeup of MEVs and highlights the various mammalian sources from which they are derived. MEVs typically contain a rich cargo of bioactive molecules, including proteins, lipids, nucleic acids (DNA, mRNA, miRNA, and other regulatory RNAs), metabolites, and pathogen-associated molecular patterns (PAMPs). These components collectively contribute to their functional roles in cellular communication, immunomodulation, and disease progression

10 and 40% [26]. MEVs provide a flexible platform for delivering therapeutic molecules to the brain because of their nanoscale size, compatibility with cell membranes, and receptor-mediated transport, which permits them to cross the blood-brain barrier (BBB) offering prospects for central nervous system (CNS) drug delivery [27]. BMEVs facilitate the preservation of protein and nucleic acid integrity during passage through the stomach and gastrointestinal tract (GIT), enabling these molecules to function locally or following their transport into the circulatory system [28]. Transmembrane and membrane-bound proteins on MEV surfaces extend the circulation time and aid in drug delivery [29]. By circumventing biological barriers and targeting specific cells or tissues with precision, these vesicles can enhance delivery efficacy and minimize off-target effects [30]. MEVs are economical and scalable drug delivery vesicles with high durability, biocompatibility, and minimal immunogenicity. They provide long-term stability, high targeting ability, substantial drug-loading capacity, enhanced solubility, and potential for synergistic therapeutic effects in disease-related applications [20, 31, 32]. Nevertheless, despite these advantages, the field faces several significant knowledge and translational gaps that must be addressed to realize the full potential of MEVs.

Current advances and persisting challenges

Despite the rapid progress in the study of MEVs, several gaps continue to constrain their full potential. A major limitation is the absence of standardized isolation and purification protocols [33]. Current methods such as ultracentrifugation, polymer-based precipitation, and SEC produce vesicles of varying purity and recovery, thereby limiting reproducibility and cross-study comparability [34]. Moreover, few investigations systematically correlate the chosen isolation method with MEV yield,

molecular integrity, or biological efficacy, underscoring the need for standardized, evidence-based workflows [13]. Drug and nucleic acid loading strategies also remain inconsistent, as commonly used methods such as incubation, sonication, or electroporation yield heterogeneous efficiencies and may compromise vesicle integrity [35]. Recent advances demonstrate the feasibility of EV mediated delivery of diverse nucleic acids, including siRNAs, miRNAs, mRNAs, and genome-editing systems; however, low loading efficiency, endosomal sequestration, and scalability remain as significant challenges, which are equally relevant to MEV platforms [8]. Finally, regulatory and industrial translation frameworks for dairy derived EVs are virtually absent, with unresolved issues concerning batch-to-batch consistency, long-term stability, and quality control [36]. Collectively, these persisting challenges emphasize the need for integrative research efforts that couple standardized isolation with advanced omics characterization, mechanistic in vivo analysis, and the establishment of regulatory and industrial guidelines to enable the reliable therapeutic and nutraceutical deployment of MEVs.

The goal of this review is to provide a thorough analysis of MEVs, focusing on their biogenesis, composition, isolation techniques, and biomedical applications. Naturally occurring MEVs have gained significant attention for their role as bioactive molecule carriers, offering promising potential in drug delivery, immune modulation, and regenerative medicine. This review summarizes their structural and functional characteristics, discusses various isolation and characterization techniques, and evaluates their biological significance. It integrates multidisciplinary insights from pharmaceutical, nutraceutical, and cosmetic research, providing a comparative framework that links fundamental molecular understanding with practical engineering and regulatory perspectives. Finally, this review highlights the current challenges in their utilization and explores future perspectives in advancing their therapeutic applications.

Biogenesis

MEV are a subset of vesicles found in milk that share the same biogenesis pathway as EVs from other cell types. Understanding their biogenesis is essential to understand their functions in health, nutrition, and potential therapeutic applications. The process starts with plasma membrane budding, forming early endosomes, maturing into late multivesicular bodies (MVBs), forming intraluminal vesicles (ILVs), and fusion with the apical plasma membrane, releasing MEVs into the extracellular space (Fig. 3). A series of regulatory processes known as "endocytosis-fusion-secretion" lead to the generation of MEVs [37]. Rab GTPases and Ral GTPases, two tiny GTP-binding proteins, and endosomal sorting complexes required to regulate their formation and release [38]. The following three essential events result in the generation of EVs.

a) EV biogenesis begins with the development of intraluminal vesicles (ILVs) into multivesicular bodies (MVBs). This involves reorganizing the

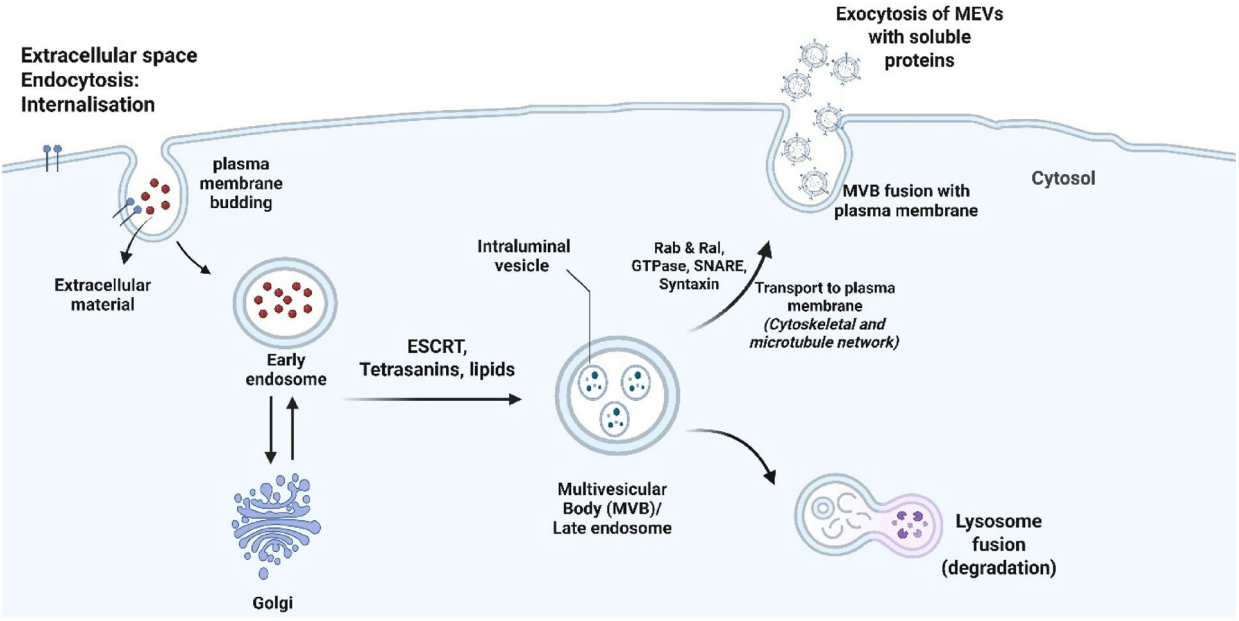


Fig. 3 Formation of MEVs, their uptake and intracellular signaling. Summarising MEV biogenesis, the major receptor-mediated uptake pathways, and subsequent intracellular signalling events. This figure provides an integrated schematic of the essential stages involved in MEV biology from their origin within cells to their functional impact on host target cells

endosome membrane to be highly enriched in tetraspanins and recruiting endosomal sorting complexes for transport (ESCRTs). Endosomes are divided into early, late, and recycled endosomes, with early endosomes being formed by invagination of the plasma membrane. The remaining early endosomes are converted into late endosomes and accumulate ILVs formed by inward budding of the endosomal membrane [12]. Notably, nucleic acid loading via cell engineering leverages endogenous EV biogenesis pathways, wherein genetic manipulation of donor cells enables selective incorporation of RNA cargo during vesicle formation, thereby enhancing nucleic acid localization and delivery efficiency [8].

b) MVBs are late endosomes with numerous small vesicles generated through the inward budding of late endosomal membranes [39, 40].
c) MVBs can either fuse with the lysosome for degradation or with the cellular membrane, releasing ILVs as EVs into the extracellular space, and are released from cells upon lysosomal degradation or membrane fusion [12].

Characteristics, composition

MEVs play an important role in intercellular communication by transporting a diverse array of bioactive molecules including proteins, lipids, nucleic acids, and carbohydrates [41]. The structural integrity of MEVs is maintained by a lipid bilayer rich in cholesterol, phospholipids, and sphingolipids, which not only ensures stability, but also facilitates efficient cellular uptake [42]. Notably, EV associated proteins, particularly membrane tetraspanins (CD9, CD63, and CD81) and RNA binding proteins, play a significant role in nucleic acid cargo sorting, localization, and stabilization within vesicles, thereby enhancing delivery efficiency. In addition, EVs carry growth factors and immunomodulatory proteins that contribute to cellular signalling and immune regulation [42]. Additionally, MEVs are enriched with microRNAs (miRNAs), mRNAs, and long non-coding RNAs (lncRNAs), which influence gene expression in recipient cells [43]. Owing to their biocompatibility, stability, and cross-species bioactivity, MEVs are gaining attention for their potential applications in therapeutics, drug delivery, and nutritional science (Table 1).

Proteins

MEVs contain proteins that are essential for their biological activity, intercellular communication, structural integrity, and therapeutic potential. These proteins contribute to cellular growth, immune regulation, tissue repair, and stability of bioactive molecules [13]. Based on their roles, these proteins can be divided into several groups.

Membrane and structural proteins

MEVs encompass a diverse range of membrane and structural proteins that are essential for their stability, biogenesis, and function in intercellular communication [44]. They play a fundamental role in the formation, trafficking, and cargo delivery of EVs, identifying them as important cellular signalling mediators and therapeutic applications [45]. Membrane proteins play essential roles in biogenesis, stability, cargo loading, and cellular interactions and consist of Tetraspanins, Milk Fat Globule Membrane Proteins, Flotillin. Tetraspanins are four transmembrane proteins that are essential for MEV biogenesis, stability, and membrane organization. They facilitate cellular recognition and uptake and play fundamental roles in the immune system through migration, proliferation, signalling, and protein trafficking. These include CD9, CD63, CD81, and CD82 [46]. CD9 plays an important role in interacting with target cells, potentially fusing them, aiding their uptake by recipient cells, and potentially regulating membrane fusion [47]. Importantly, they serve as anchoring scaffolds for RNA binding proteins, enabling enhanced encapsulation and stabilization of therapeutic nucleic acids within EVs [8]. CD63 contributes to stability, biogenesis, cargo sorting, and interaction with target cells, influencing cell-to-cell communication [48]. CD81 Facilitates MEV trafficking, cell adhesion, signal transduction, cell-to-cell communication, and transport of molecules [49]. CD82 plays an essential role in biogenesis, cargo selection, cell communication, and immunological signalling [50].

MFGM is a multilayered structure found in mammalian milk that is predominantly composed of phospholipids and proteins. They are important for membrane stability, cellular communication, immune modulation, and bioactive molecule transport, and have been extensively studied for their nutritional and therapeutic properties [51]. These include Butyrophilin, Milk Fat Globule-EGF Factor 8 (MFG-E8), and lactadherin. Butyrophilin (BTN1A1, BTN3A1): Controls immunological responses and lipid secretion [52]. Milk Fat Globule-EGF factor 8 (MFG-E8) promotes tissue healing, immunological control, and MEV cell interaction [53]. Lactadherin: Contributes to immunological regulation and gut health and improves phagocytosis [25].

In addition to tetraspanins and MFGM proteins, MEVs contain several other important protein groups that support structural integrity, biological activity, and function in intercellular communication. Notable among these are flotillins and annexins, each of which play a distinct role in EV function. Flotillins (Flotillin-1 and Flotillin-2) facilitate vesicle production, membrane stability, and endocytic activities [54]. Annexins (Annexin A1, A2, A5) play roles in membrane fusion, EV trafficking, and vesicle stability [55].

Structural proteins

Table 1 Overview of MEV composition

Category		Representative molecules	Major functions	Biological Roles/Action Pathways	Role in MEV Applications	Reference
Proteins (60–80%)	Membrane & Structural Proteins	Tetraspanins (CD9, CD63, CD81, CD82)	Involved in EV biogenesis, cargo sorting, membrane fusion, and cell adhesion.	• Integrin–FAK–Src pathway • EGFR–MAPK/ERK signaling • PI3K–Akt pathway • Immune synapse signaling	Serve as EV surface markers; facilitate cellular uptake and targeting in drug delivery	[98–101]
		Milk Fat Globule Membrane Proteins (Butyrophilin, MFG-E8, Lactadherin)	Maintain membrane stability, immune modulation, and phagocytosis	• Integrin αvβ3/β5–PI3K–Akt pathway (Lactadherin) • T cell co-stimulation and immune regulation (Butyrophilin) • Oxidative stress pathways (Xanthine oxidase)	Enhance immunological activity and gut health; potential nutraceutical targets	[102, 103]
		Flotillins (Flot-1, Flot-2)	Involved in endocytosis and vesicle formation	• Caveolin-independent endocytosis pathway • MAPK/ERK signaling • Wnt/β-catenin pathway	Regulate EV trafficking and release; used as EV isolation markers	[104–106]
		Annexins (A1, A2, A5)	Membrane repair, fusion, and vesicle stability	• Phospholipase A2 inhibition pathway • TGF-β/Smad signaling • NF-κB suppression • EGFR-mediated endocytosis	Enhance MEV stability and delivery efficiency	[107–109]
		ESCRT proteins (Alix, TSG101)	Multivesicular body (MVB) formation and cargo sorting	• ESCRT-dependent exosome biogenesis pathway • Ubiquitin–TSG101–Alix pathway • Rab GTPase (Rab27/11) trafficking pathway	Essential in EV biogenesis; targets for controlled cargo loading	[110, 111]
		Cytoskeletal Proteins (Actin, Tubulin)	Structural support and intracellular trafficking	• Rho GTPase signaling (RhoA, Rac1, Cdc42) • PI3K–Akt pathway • Integrin–FAK pathway	Maintain vesicle shape and transport functions	[112, 113]
		Heat Shock Proteins (HSP70, HSP90)	Protein folding, stress response, and immune regulation.	• Toll-like receptor (TLR2/4–NF-κB) signaling • MAPK–ERK stress response • Antigen presentation via MHC pathways	Mediate immune modulation; serve as biomarkers and delivery stabilizers	[114, 115]
	Signalling & Functional Proteins	Growth Factors (TGF-β, IGF, EGF)	Promote cell proliferation, differentiation, and immune regulation	• TGF-β/Smad2/3 pathway • PI3K–Akt–mTOR pathway •MAPK/ERK cascade •JAK–STAT signalling	Therapeutic applications in tissue regeneration and gut development	[116, 117]
		Cytokines (ILs, TNF, Lactoferrin)	Immune modulation and antimicrobial effects	• JAK–STAT3 signaling (IL-6, IL-10) • NF-κB pathway (TNF-α) • TLR4–MyD88 signaling (Lactoferrin)	Modulating immune responses, regulating inflammation, and promoting cell growth and survival	[118, 120]
		Enzymes (Kinases, Proteases, Lipases)	Signal transduction, metabolism, and protein processing	• MAPK/ERK and PI3K–Akt pathways (Kinases) • MMP-mediated ECM remodeling (Proteases) • Lipid metabolism and arachidonic acid pathways (Lipases)	Support metabolic regulation and bioactive functionality	[121, 122]
	Adhesion & Cell Interaction Proteins	Integrins, Glycoproteins	Mediate MEV binding and uptake via receptor-ligand interactions	• Integrin–FAK–Src signaling • PI3K–Akt pathway • MAPK/ERK pathway	Essential for targeted delivery and cellular uptake mechanisms	[123, 124]

Table 1 (continued)

Category	Representative molecules	Major functions	Biological Roles/Action Pathways	Role in MEV Applications	Reference
Lipids (30–45%)	Phosphatidylcholine (PC)	· Membrane stability and vesicle curvature	• Phospholipase D–choline signaling Membrane fusion and lipid raft regulation	Maintains MEV integrity and supports drug encapsulation	[125, 126]
	Phosphatidylserine (PS)	· Immune evasion, macrophage recognition	• Phagocytic receptor–mediated uptake (TIM, TAM receptor pathways) Annexin V binding and apoptotic mimicry signaling	Promotes uptake and modulates immune tolerance	[127, 128]
	Phosphatidylethanolamine (PE)	· Membrane fusion and flexibility	• Autophagy regulation via LC3–PE conjugation pathway Membrane fusion and trafficking pathways	Supports MEV fusion with recipient cells	[129]
	Phosphatidylinositol (PI)	· Cell signalling and membrane trafficking	• PI3K–Akt–mTOR pathway • PLC–IP_3–Ca^{2+} signaling Rab GTPase-mediated endosomal sorting	Enhances communication and intracellular signaling	[130, 131]
	Sphingomyelin (SM), Ceramides, Cholesterol	· Membrane rigidity, domain formation, and stability	• Sphingomyelinase–ceramide pathway • NF-κB and apoptosis signaling • Neutral sphingomyelinase (nSMase2)–ceramide pathway - JNK and NF-κB stress pathways	Affect vesicle curvature, storage stability, and delivery efficiency	[132]
	Lysophospholipids	· Membrane dynamics and receptor activation	• LPA receptor (LPAR1–6)–Rho/ROCK signaling • GPCR-mediated Ca^{2+} mobilization	Facilitate MEV–cell interaction and bioactive lipid signaling	[133]
Nucleic Acids (2–5%)	mRNAs	· Encode functional proteins influencing recipient cell function	• Translation and protein synthesis pathways (e.g., mTOR, EIF2) Cell cycle and metabolic regulation	Potential to modulate protein synthesis in target cells	[134]
	miRNAs (e.g., miR-148a, miR-21, miR-200a)	· Post-transcriptional regulation of genes; modulate immune and developmental pathways	• DNMT1 and TGF-β/Smad pathway • PTEN–PI3K–Akt–mTOR pathway • ZEB1/2–EMT regulation • Collagen synthesis suppression via TGF-β NF-κB and immune modulation	Regulating intestinal maturation, immune modulation, inflammation resolution, and suppression of proinflammatory pathways	[135, 136]
	lncRNAs NEAT1, MALAT1	· Gene expression control and cell proliferation	• NEAT1: Paraspeckle–STAT3–NF-κB signaling MALAT1: Wnt/β-catenin and PI3K–Akt–mTOR pathways	Modulating gene expression, inflammation, and cellular stress responses, thereby contributing to their immunoregulatory and regenerative functions.	[137, 138]
	rRNAs, tRNAs, tRNA-derived fragments (tRFs)	· Maintain translation machinery; regulate gene expression	• RNA interference and gene silencing pathways Immune modulation via TLR3	Regulating protein synthesis, modulating immune responses, and influencing gene expression	[139, 140]
	DNA (nuclear and mitochondrial)	· Reflects cellular origin; may modulate recipient gene expression	• cGAS–STING pathway (innate immune activation) TLR9 signaling (endosomal DNA sensing)	Modulate gene expression, cellular metabolism, and immune responses	[141, 143]

The morphological integrity, stability, and biological activity of MEVs are influenced by their structural proteins. These proteins aid in the structure of the MEV membrane and play vital roles in intracellular trafficking, cargo protection, and vesicle formation. Heat shock proteins (like HSP70 and HSP90), cytoskeletal components (like actin and tubulin), and ESCRT-associated proteins (such as Alix and TSG101) are some of the most common structural proteins in MEVs.

Cytoskeletal components (Actin and Tubulin) play an important role in cell formation, structural integrity, and functional activity, including cell signalling, cell adhesion, immune regulation, biogenesis, trafficking, and intercellular communication [56]. Actin contributes to the formation and trafficking of these vesicles, as well as

potentially influencing their cargo and activity, contributing to preserving the vesicle's structure and facilitating its interactions with other cells and tissues [57]. Tubulin play significant role in cell structure, movement, and division [58].

Heat Shock Proteins (HSP70 and HSP90) play a role in protein folding, stress response, and protection from denaturation. HSP70 preserves the intestinal epithelial barrier function by stabilizing tight junctions and potentially affecting immune responses [59]. HSP90 assists in protein folding, stability, and trafficking within MEV. It also plays a role in the membrane deformation [60].

ESCRT-associated proteins (such as Alix and TSG101) participate in vital cellular functions, formation of multivesicular bodies (MVBs), endosomal membrane budding and abscission, and MEV cargo selection by interaction with syndecan [61]. Alix plays a important role in the biogenesis and sorting of cargo [62]. TSG101 is involved in sorting and budding of cellular cargo into multivesicular endosomes [63].

Signalling and functional proteins

These proteins modulate immune responses, facilitate cell signaling, contribute to nutritional value, exhibit anti-inflammatory and antimicrobial properties, and enhance the functional potential of MEV. It includes Growth Factors, Cytokines, Immunomodulatory Proteins, and Enzymes [64]. Growth Factors (TGF-β, IGF, and EGF) promote cell proliferation, immune system development, gut barrier function, inflammation regulation, neurodevelopment, and drug delivery [65]. TGF-β help in developing the intestinal barrier function, stimulating IgA production, and promoting mucosal immunity [66]. IGF promotes cell growth and development and influences intestinal health [67]. EGF promotes intestinal development and maturation [68]. Cytokines and Immunomodulatory Proteins (lactoferrin, tumor necrosis factor receptor, and interleukins) contribute to immune regulation, anti-inflammatory responses, and antimicrobial activity [69]. Lactoferrin promotes immune system development, anti-inflammatory effects, potential cancer anti-cancer activity, gastrointestinal development, immunity protection, and microbiota establishment, potentially reducing infection risk [70]. Tumour Necrosis Factor exerts anti-inflammatory effects [71]. Interleukins contribute to immune modulation and Anti-inflammatory activity [48].

Enzymes (Kinases, Proteases, Lipases) are involved in cellular signalling, metabolic regulation, and protein processing within recipient cells [48]. Kinases modulates the functions of MEVs [72]. Proteases contribute to both digestion and immune function [73]. Lipases contribute to both the breakdown of triglycerides and modification of fatty acid chains [74].

Adhesion and cell interaction proteins

They facilitate the uptake and delivery of cargo by mediating interactions between MEV and target cells [75]. Integrins mediate the binding of MEVs to target cells and facilitate internalization [76]. Glycoproteins are involved in receptor-mediated uptake and immune signalling [77].

Lipid composition of the MEVs

MEVs are encapsulated by a lipid bilayer that ensures their structural integrity and protects their bioactive cargo during extracellular transport. The specific lipid composition of MEVs plays a pivotal role in determining their stability, cellular uptake, and biological functionality. Compared with other milk fractions, MEVs exhibit a distinct lipid profile, both in composition and concentration, and are often enriched in lipids that promote membrane curvature, vesicle budding, and efficient MEV formation [78].

Phospholipids

Phospholipids are the principal components of the MEV membranes. This phospholipid bilayer not only maintains the structural stability of the internal cargo but also contributes to the bioactivity of vesicles, thereby facilitating their role in intercellular signalling and communication [79]. Phosphatidylcholine (PC) is the most abundant phospholipid that contributes to membrane stability, influences cell signalling, facilitates cargo delivery, and potentially influences cell-to-cell communication [80]. Phosphatidylserine (PS) is mostly found on the outer leaflet of it is important for both immune evasion and innate immunity. It draws in macrophages, makes it easier for them to absorb them, and may change immune responses or allow viruses to enter the cells [80]. Phosphatidylethanolamine (PE) is essential for MEV membrane stability and cell interaction, forming a lipid bilayer and protecting internal components [81]. Phosphatidylinositol (PI): PI, along with other lipids, contributes to the structure and function of MEVs and participates in bioactive molecule transport and intercellular communication [82].

Sphingolipids

Sphingolipids are complex lipids essential for the structure and function of MEVs. They contribute to the rigidity, stability, and cellular signalling of the MEV membrane [83]. Sphingomyelin (SM) is an important structural component of cell membranes and neuronal lipid bilayers and is involved in cell proliferation, differentiation, stress response, and migration [84]. Ceramides are essential lipids that shape the MEV membrane, influencing their ability to encapsulate and transfer molecules between cells [85]. Cholesterol is essential for structural integrity, functional interactions, and cargo delivery efficiency, enhancing lipid bilayer stability and facilitating fusion with recipient cell membranes [86].

Lysophospholipids

Lysophospholipids, a subclass of phospholipids, are absent fatty acid chains that play significant roles in membrane dynamics, bioactivity, cell signalling, and interactions with recipient cells in MEVs [87].

Nucleic acid composition of the MEVs

MEVs carry a diverse range of nucleic acid components, encompassing both coding and noncoding RNAs and DNA. Furthermore, the presence of both nuclear and mitochondrial DNA has been identified in MEVs [88]. EVs have been most extensively investigated for the transport of small RNAs, particularly siRNAs and miRNAs, owing to their intrinsic biocompatibility, ability to cross biological barriers, and capacity for cytosolic cargo delivery. However, delivery efficiency is highly dependent on EV origin, loading strategy, surface composition, and intracellular trafficking pathways, which are equally relevant to MEVs [8]. Mechanistically, nucleic acid loading into EVs occurs via two principal routes: (i) cell engineering based approaches, which exploit endogenous EV biogenesis and RNA-sorting machinery through genetic or transcriptional manipulation of donor cells, and (ii) direct EV engineering, in which isolated vesicles are post-synthetically modified through electroporation, chemical conjugation, or hydrophobic insertion [8]. Localization and intracellular fate are significant determinants of nucleic acid bioactivity. Several studies demonstrate that unmodified EVs may suffer from endo-lysosomal sequestration, leading to suboptimal gene silencing. This limitation has been successfully addressed through membrane engineering strategies, including viral fusogenic proteins (e.g., VSV-G), pH-responsive polymers, and EV synthetic hybrid systems that promote direct cytosolic release [89]. Importantly, MEVs have already shown inherent stability in gastrointestinal conditions and enhanced mucus penetration, positioning MEVs as particularly attractive carriers for oral and mucosal nucleic acid delivery. PEGylation, ligand conjugation (e.g., folic acid), and lipid insertion strategies reported for other EV systems are directly translatable to MEVs to improve tissue localization, cellular uptake, and endosomal escape [90]. Delivery efficiency varies substantially across EV platforms, with reported encapsulation efficiencies for small RNAs typically below 30% in native EVs, and even lower for large nucleic acids such as mRNAs and CRISPR/Cas systems [91]. MEVs, however, offer distinct advantages in scalability and batch to batch consistency due to their natural abundance and established dairy infrastructure. Studies employing MEVs for siRNA delivery have demonstrated that surface functionalization and hybridization with synthetic lipids can significantly enhance nucleic acid stability, gastric resistance, and transfection efficiency, underscoring the feasibility of MEVs as clinically relevant carriers. Despite advances, several challenges remain for MEV based nucleic acid delivery, including standardized large scale isolation, precise control over cargo loading, avoidance of co-isolated contaminants, and comprehensive evaluation of biodistribution and long term safety. Moreover, while MEVs are generally regarded as safe, systematic studies examining their immunogenicity following repeated nucleic acid loading and surface modification are still required.

RNAs

RNAs in MEVs can regulate gene expression, affect cellular functions and development, and facilitate absorption and uptake. These include messenger RNAs (mRNAs), microRNAs (miRNAs), long non-coding RNAs (lncRNA), ribosomal RNAs (rRNAs), and transfer RNAs (tRNAs) [92]. mRNAs play vital roles in protein synthesis, intercellular communication, and gene expression regulation, potentially influencing cell function [93]. miRNAs act as regulatory molecules, transferring genetic information and influencing biological processes in recipient cells, including immune responses and development [94]. lncRNAs regulate cell-to-cell communication, gene expression, immune function, and cell proliferation [95]. rRNAs regulate gene expression and intercellular communication, protecting them from degradation and allowing them to be transported to recipient cells for cellular processes [96]. tRNAs regulate MEVs by supplying tRNA-derived fragments (tRFs) that can be absorbed and function in recipient cells, potentially influencing gene expression and cellular processes [92].

DNA

DNA is essential for cell-to-cell communication and gene expression and is involved in intercellular signalling [97].

Mechanisms of MEV uptake

The uptake mechanisms of EVs and MEVs share similarities, with endocytosis being the primary route for MEV internalization. MEVs enter recipient cells through various endocytic pathways, including clathrin-mediated endocytosis (CME), caveolin-dependent endocytosis (CDE), macropinocytosis, phagocytosis, and direct membrane fusion (Fig. 4). The uptake process is influenced by MEV surface proteins and the recipient cell type. Once internalized, MEVs release their bioactive cargo including proteins, RNAs, and lipids into the cytoplasm, where they influence cellular signalling, immune responses, and gene expression.

Clathrin-mediated endocytosis (CME)

Clathrin-mediated endocytosis (CME) is an essential endocytic pathway in mammalian cells that oversees cell surface signalling, plasma membrane composition, and transmembrane receptor uptake (Fig. 4) [137]. CME plays a significant role in MEV uptake by intestinal epithelial

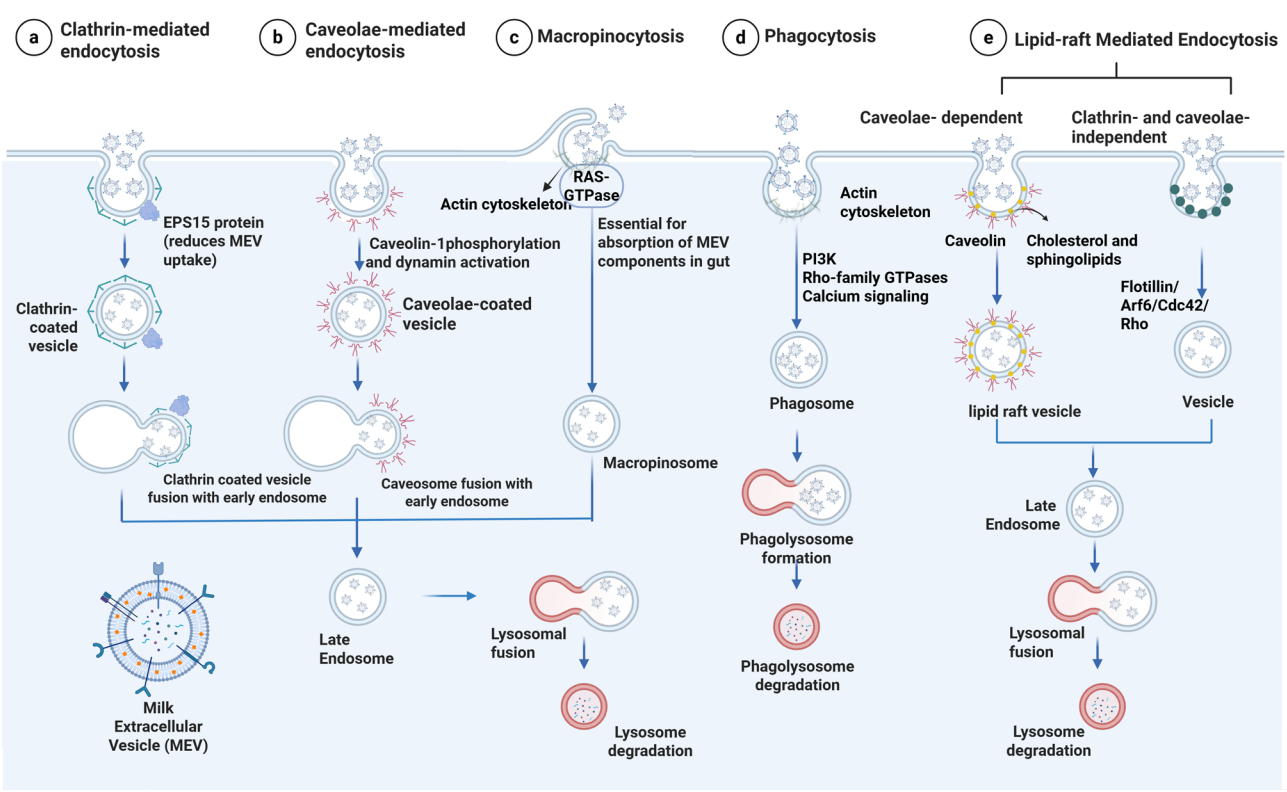


Fig. 4 Internalization mechanism of MEVs This figure illustrates the principal endocytic pathways through which MEVs enter host cells. MEVs exploit multiple internalization routes depending on their size, surface composition, and the receptor landscape of the target cell. The schematic highlights key mechanisms such as clathrin-mediated endocytosis, a highly regulated pathway involving cargo recognition and vesicle formation through clathrin-coated pits, and caveolin-mediated endocytosis, which utilizes lipid raft–rich caveolae for selective uptake of vesicles and associated signalling molecules

cells (enterocytes and M cells) [138]. Clathrin-coated vesicles internalize MEVs through receptor-ligand interactions. Once inside, vesicles uncoat and fuse with endosomes for cargo release [139]. Epidermal growth factor receptor pathway substrate clone 15 (EPS15) inhibits CME and reduces MEV uptake, confirming its importance in MEV transport [140].

Caveolin-dependent endocytosis (CDE)

Caveolae are lipid-rich domains that facilitate MEV uptake by interacting with lipid rafts and specific receptors (Fig. 4) [141]. Caveolae clustering leads to vesicle formation and internalization, followed by fusion with early endosomes [142]. This selective pathway is regulated by caveolin-1 phosphorylation and dynamin activation, making it essential for absorption of bioactive MEV components in the gut [143].

Macropinocytosis

Macropinocytosis is a cellular process in which cells ingest extracellular fluid and particles through ruffles, creating macropinosomes via actin cytoskeleton remodelling (Fig. 4) [144]. MEVs can also be internalized through macropinocytosis, a bulk uptake mechanism that involves actin-driven membrane ruffling [145]. Extracellular fluid and MEVs are engulfed by macropinosomes, which are large vesicles that subsequently fuse with endosomes and lysosomes to release or degrade cargo [146]. Macropinocytosis, regulated by Ras-family GTPases, plays a significant role in nanocarrier accumulation, making it a promising route for therapeutic MEV applications [147].

Phagocytosis

Phagocytosis is a cellular process that involves immune cells such as macrophages, neutrophils, and monocytes to eliminate foreign particles, dead cells, and debris (Fig. 4) [148]. It involves phagocytic receptors on immune cells that recognize surface markers on MEVs, and opsonization enhances this recognition [149]. Actin cytoskeleton remodelling occurs when phagocytes engulf MEVs and is regulated by PI3K, Rho-family GTPases, and calcium signalling [150]. Engulfed MEVs are enclosed in phagosomes, which fuse with lysosomes to form phagolysosomes [151]. Phagocytosis influences macrophage polarization, inflammatory responses, antigen presentation, immune tolerance, and intestinal homeostasis [148]. Phagocytosis plays an important role in targeting drug

delivery systems to immune cells, but strategies to evade immune detection are required for therapeutic applications involving nonimmune cells [152].

Lipid raft-mediated endocytosis

Lipid raft-mediated internalization encompasses various endocytic pathways, including caveolae-dependent uptake, and clathrin- and caveolae-independent mechanisms regulated by flotillin, Arf6, Cdc42, and Rho A (Fig. 4). Lipid raft-mediated endocytosis is essential for processes such as cell signalling, viral entry, and disease association [153]. MEVs interact with lipid rafts cholesterol and sphingolipid-rich membrane domains to enter cells via flotillin- and caveolae-dependent mechanisms. This pathway plays an important role in cell signalling, viral entry, and disease-related processes [154]. The regulation of raft-dependent endocytosis by tyrosine kinase inhibitors highlights their importance in MEV uptake and function [155].

Direct membrane fusion

MEVs can fuse with recipient cell plasma membranes, release the cargo into the cytoplasm, bypass endocytic pathways, and potentially trigger rapid signalling responses. Direct membrane fusion involves binding of MEVs to recipient cell plasma membranes, facilitated by surface proteins such as tetraspanins and ligands. The proximity of the lipid bilayers between MEVs and target cells is important for initiating fusion. Close contact forms a fusion stalk, which forms pores for lipid and protein exchange. Once stable, the pores expand, releasing MEV into the cytoplasm of the recipient cells [156].

Pharmacokinetics and biodistribution of MEVs

Understanding the ADME processes of MEVs is essential for optimizing their clinical application. MEVs are internalized by various cell types via endocytosis, phagocytosis, macropinocytosis, or direct plasma membrane fusion [160]. Surface glycoproteins on MEVs facilitate endocytosis into intestinal epithelial cells, allowing for the transport of nutrients and bioactive molecules through specific proteins, such as CD63, CD81, and integrins [161]. Kusuma et al. found that endothelial cells internalise MEVs via temperature-dependent endocytosis, with uptake decreasing by 80% at lower temperatures and by 45–80% when surface proteins were removed. The removal of MEV surface proteins by enzymes decreases their uptake by human vascular endothelial cells, indicating that protein-ligand interactions are essential for effective transport [162]. Glycoproteins in MEVs interact with lectins and intestinal epithelial cells, enhancing their absorption [163]. A fraction of dietary MEVs may enter systemic circulation, highlighting their potential role in intercellular communication, nutrient absorption, and drug delivery [70]. Badar J et al. study suggests that the biodistribution of MEVs is influenced by intrinsic characteristics such as size, charge, lipid composition, and membrane rigidity [164, 165]. Smaller vesicles have enhanced permeability, whereas the lipid composition affects membrane fusion and stability [166]. The presence of phosphatidylserine and cholesterol in MEV membranes facilitates uptake by macrophages and dendritic cells, impacting their immunomodulatory properties [13]. Physiological factors, such as pH, enzymatic degradation, microbiota composition, and immune responses, also regulate MEVs stability and absorption [167]. Tissue distribution of MEVs is associated with issues of therapeutic efficacy and safety, such as tissue accumulation and unfavorable outcomes [168]. MEVs are eliminated via splenic filtration via phagocytosis, hepatic clearance through lysosomal degradation and biliary systems, and renal excretion [169]. The body eliminates absorbed MEVs through the metabolism and excretion of sugars, cyclic alcohols, carnitines, carboxylic acids, amino acids, vitamins, lipids, nucleotides, and nucleosides in the MEV metabolome [170]. MEVs release cargo into the bloodstream, which subsequently undergoes endocytic pathways for degradation and recycling. However, MEVs persist in tissues such as the liver and spleen for over 24 h, with the kidneys, liver, and spleen being most affected by their clearance [171]. The pharmacokinetics and biodistribution of MEVs have been investigated using bioluminescence, with emphasis on the influence of parent cells or EV sources.

Stability parameters of MEVs

The stability of MEVs is an essential factor for their potential use in therapeutic applications, particularly drug delivery systems. Various factors, including physical, chemical, and biological conditions, affect the stability of MEVs [20]. A comprehensive understanding of these factors is essential for optimizing the processes of isolation, formulation, and storage, and ensuring both the efficacy and safety of MEVs for clinical applications.

Temperature sensitivity

The stability of MEVs is significantly influenced by temperature [172]. Komuro et al.. indicated that MEVs are most stable when maintained at low temperatures, typically at −80 °C–4 °C, which preserves both their payload and structural integrity [173]. Görgens A et al.. revealed that keeping EVs in PBS throughout time reduces recovery rates, especially for pure samples. PBS-HAT buffer, enhanced with trehalose and human albumin, which enhances recovery when used as a diluent, stability during freeze-thaw cycles, and EV preservation for samples kept at −80 °C [174]. At higher temperatures, vesicles may aggregate, degrade, or fuse, resulting in a loss of

functional ability. The stability of MEV is also affected by freeze-thaw cycles, which may alter the size, integrity, and bioactivity of the vesicles. Consequently, appropriate cold storage is necessary to maintain its stability [175].

pH and ionic strength

Environmental pH and ionic strength substantially influence the stability of MEVs. They remain stable within the physiological pH range of 7.2–7.4; however, deviations from this range can lead to destabilization, including membrane rupture or alterations in surface properties [176]. Midekessa G et al. concluded that ionic strength is important for maintaining electrostatic interactions between vesicles and their payload, and an optimal balance of ionic concentration is essential for structural integrity and functionality [177, 178].

Surface charge and composition

The surface charge of MEVs, which is determined by their lipid and protein compositions, influences their interactions with the surrounding environment. According to Hallal et al., EVs are composed of glycoproteins and their negative surface charge causes electrostatic repulsion, which prevents aggregation and dispersion under physiological conditions [179]. Specifically, phospholipids and proteins contribute to a negative surface charge that enhances the stability in suspension and inhibits aggregation [180]. Alterations in lipid and protein contents during separation or storage may affect the surface charge, potentially impacting vesicle stability and their interaction with target cells or tissues [181].

Lyophilization and cryopreservation

Lyophilization and Cryopreservation techniques are frequently employed to preserve MEVs during extended storage. Cryopreservation is a technique that employs cryoprotectants to safeguard vesicles against damage during the freezing and subsequent thawing processes. This method requires freezing of vesicles in conjunction with protective substances such as Trehalose, Mannitol, Methionine, Sucrose. The study by Walker SA et al. compared phosphate-buffered saline/PBS and a cryoprotective 5% sucrose solution for EV storage. These findings indicated that the buffer used for storage at-80 °C had a substantial impact on EV size, concentration, transmembrane proteins, and molecular surface extensions. This suggests that biocompatible cryoprotectants such as sucrose should be employed in EV research [182]. The process of lyophilization, commonly referred to as freeze-drying, facilitates prolonged storage in a dehydrated state by eliminating water from the MEVs [183]. Charoenviriyakul et al.. developed a room-temperature storage method for EVs by comparing their properties with those stored at −80 °C. Lyophilization without cryoprotectants caused aggregation of EVs, while lyophilization with trehalose prevented aggregation [184].

Storage media and additives

The selection of storage media and stabilizing agents influences MEV stability. According to Walker et al., buffers containing substances, such as trehalose (25 mM) or sucrose (5%), have been demonstrated to enhance the preservation of MEVs during storage [182]. These stabilizers aid in maintaining vesicle integrity, preventing aggregation, and protecting against environmental stressors such as oxidative damage. Storage solutions can be fortified with antioxidants, such as Glutathione, Trolox, to counteract oxidative deterioration and may potentially impair the performance of MEVs [185].

Size distribution and aggregation

Two significant indicators of stability are the size distribution and aggregation state of MEVs. The size distribution of well-characterized and stable MEV preparations typically falls within a narrow range, with vesicle diameters between 30 and 150 nm. Aggregation may lead to a reduced targeting efficacy and altered bioactivity [174].

Isolation methods

Isolation of MEVs is an important process for examining their architectural features, molecular makeup, and physiological roles, as well as exploring their potential utility in both treatment and diagnostic procedures. Numerous separation techniques have been devised, each exhibiting specific strengths and constraints that leverage physical characteristics such as size, density, and surface markers. The methodologies for isolating MEVs (Table 2) include density gradient, ultracentrifugation (UC), differential centrifugation, precipitation, ultrafiltration, immunoaffinity, microfluidics, and size-exclusion chromatography [186].

Ultracentrifugation (UC)

Ultracentrifugation is a commonly used method for isolating MEVs from biological fluids [187]. This process entails three to four cycles of low-speed and high-speed centrifugation (4 °C) at 5000 × g and 100000 × g for shorter and longer periods, respectively, to segregate larger vesicles and precipitate MEVs as pellets [188]. As the distance of individual MEVs from the pellet fluctuates, differential ultracentrifugation (DC) cannot accomplish absolute separation of MEVs based solely on size [189]. Additionally, DC may cause MEVs to cluster, co-isolate non-EV components, and sustain damage during the last stage of ultracentrifugation [190].

Table 2 Methods of MEV isolation: principles and discussion

Method of isolation	Principle	Discussion	Limitations	Reference
Ultracentrifugation	The technique involves sedimentation, where speed is determined by sedimentation coefficient, rate-zonal and isopycnic separation uses size and mass, often with density gradient	• Provides high purity, reproducibility for biomarker discovery and therapeutic development • Applicable to various biological fluids • Allows isolation of specific subpopulations based on size and density • Lower operating costs compared to commercial kits	Risk of EV damage, aggregation, and equipment intensive	[212]
Density gradient ultracentrifugation	Particles move in a centrifugal field within a density gradient medium, where denser particles descend and less dense ones rise. Specific layers can be collected to isolate pure populations	• Improves purity through efficient separation • Enhances recovery rates with biocompatible media • Compatibility with downstream applications • Scalability for larger-scale isolations	Low yield, long processing time, potential vesicle loss, and partial contamination from milk proteins.	[213]
Precipitation	The precipitation principle for MEV isolation involves aggregating MEV in a solution with precipitating agents, causing dehydration and reduced solubility. Visible particles are separated by centrifugation and then resuspended for further purification	• High yield for downstream analyses • Cost-effective with inexpensive, widely available reagents • Selectivity based on size and surface properties • Scalability for small and large sample volumes • Minimized sample handling reduces the potential for loss or contamination	Co-isolation of non-EV proteins, lipoproteins, and polymer residues, leading to lower purity and potential interference in downstream analyses	[214]
Ultrafiltration	The sample preparation and membrane filtration to isolate MEVs, retaining them on one side while eliminating smaller contaminants. These contaminants can subsequently be concentrated through reduction of the filtrate volume or resuspension in a smaller buffer volume	• Provides high recovery yield • Quick, straightforward process • Less expensive than specialized equipment • Scalable based on sample volume • Gentle process maintains MEV integrity • Enhances purity by separating MEV from smaller contaminants	Membrane clogging possible	[215]
Immunoaffinity	It uses antibodies, aptamers, or lectins to target surface proteins on MEVs, capturing specific subpopulations based on origin or functional characteristics immobilized on a solid support	• Targets surface proteins for pure MEV samples • Allows specific subpopulations of MEVs for study • Compatibility with downstream applications for proteomic analysis and functional assays	High cost and low yield, as it targets only specific surface markers, potentially excluding heterogeneous EV populations	[216]
Microfluidics	Microfluidics uses miniaturized systems to isolate MEV from biofluids. It involves efficient separation using principles like size-based, hydrodynamic focusing, viscoelastic forces, active, passive, and sequential filtering, enhancing purity and recovery rates	• Requires smaller sample volumes for limited samples • Isolates MEVs quickly for timely results • Suitable for point-of-care applications and high-throughput screening • Lowers operational costs • Enhances reproducibility and reduces human error	Low processing throughput, making it unsuitable for large-volume samples and prone to device clogging due to the high protein and fat content of milk	[217]
Size-exclusion chromatography	SEC is a method for separating MEVs from other contaminants based on size, using a stationary phase with porous beads. It allows smaller molecules to enter while preventing larger particles like MEVs	• Removes most soluble contaminants. • Simplifies process and reduces complexity • Maintains structural integrity and biological activity • Scalable and flexible for larger sample volumes. • Cost-effective with lower initial setup costs than ultracentrifugation	Co-isolation of proteins possible	[218]

Size exclusion chromatography

Size exclusion chromatography (SEC) is an effective, cheap, and rapid method for the separation of MEVs from their soluble constituents, including proteins and high-density lipoproteins (HDL) [191]. The idea behind SEC is that macromolecules are flushed along the gel pores with a mobile phase because they cannot pass through the gel apertures [192]. The process entails the elution of small molecules using an eluent from the porous matrix, while macromolecules are flushed along the pores of the gel using a mobile phase [192]. Non-EV components, including viruses, protein aggregates, large proteins, and

chylomicrons, are co-isolated with EVs, including high-molecular-weight proteins, LDL, chylomicrons, cells, and cell detritus [193, 194]. Combining different EV-containing fractions enhances recovery but reduces purity [196].

Ultrafiltration (UF)

MEVs can be separated from soluble components by ultrafiltration, which involves either applying pressure or centrifuging the filter [197]. Compared to DC, this method requires less time; it takes approximately 20 min to concentrate more than 100 mL of the material. This is useful for MEV concentration because it can recover up to 80% of MEVs and concentrate them up to 240 times [198]. MEVs can be quickly extracted from materials via ultrafiltration, also known as size-based exclusion, which applies size-exclusion criteria or preset molecular weights to a membrane filter [195].

Immunocapture assays

Immunocapture assays utilize surface-immobilized monoclonal antibodies to specifically capture MEVs that express certain ligands, predominantly proteins [199]. In immunoaffinity chromatography, antibodies are covalently attached to filters or matrices, enabling them to bind to surface proteins or antigens on MEVs that are necessary for immunocapture [200]. Immunocapture tests recover non-EV proteins and a repository for these proteins is available [195]. However, due to variable extraction methods, high costs, and low purity and yield of MEV, no kit can optimally extract MEVs from mixed materials [201].

Precipitation

Polyethylene glycol, a benign and non-denaturing polymer, is frequently used in MEV precipitation kits to identify biomarkers associated with MEVs [202]. These kits do not require special equipment and can be used for both low and high sample volumes [203]. However, as precipitation primarily focuses on concentration, it is unsuitable for detecting MEV-associated biomarkers [204]. The optimal method for detecting MEVs of 50 nm or larger should have established detection limits, a defined sample volume, and the capability to identify each MEV immunophenotype. (225,226). The precipitation method for isolating MEVs involves several approaches: the application of protamine sulfate, introduction of saline solutions, elimination of polymeric substances, or manipulation of aqueous environments to modify MEV solubility or dispersal characteristics [201].

Microfluidic technology

The application of microfluidic technology has significantly improved the separation of MEVs [206]. These techniques include filter-based extraction, tag-free separation using microfluidics in conjunction with dielectric electrophoresis and ultrasonic waves, and immunoaffinity-based extraction using biomarkers such as antibodies [207]. Nanoplasmonic-enhanced scattering (nPES) may be used to identify all MEVs in a sample by employing antibodies against biological markers CD81, CD63, and CD9 [208]. This method efficiently produces MEVs and is easy, rapid, cost-effective, and scalable [209]. Techniques such as tangential flow filtration, deterministic lateral displacement array, hydrophobic interaction chromatography, and flow field-flow fractionation offer opportunities for MEV enrichment. To further summarize the choice of suitable isolation methods, a decision flowchart has been provided (Fig. 5).

Drug loading methods

Drugs can be incorporated into MEVs via transfection, in situ synthesis and assembly, passive drug loading (incubation), or active drug loading (Fig. 6) [106]. MEVs should be made to undergo preliminary cargo unloading to create adequate space before starting the main free-loading operation. Strict monitoring of conditions such as the structural integrity of MEVs and proper handling and storage is essential for preserving MEV stability, ensuring precise drug release, and maintaining functional efficacy before, during, and after off-loading for optimal therapeutic delivery [26]. The most straightforward method enhances drug diffusion into MEVs by incubating them with the drug at a set temperature for a specified duration, using a concentration gradient [118]. Transfection enables the consistent incorporation of proteins, peptides, and nucleic acids into MEVs by transducing specific plasmids into cells using transfection reagents, resulting in ectopic production and subsequent loading of the desired cargo into MEVs [119]. There are two primary issues with this strategy: contamination and poor loading efficiency [101]. To enable drugs to enter MEVs, active drug loading involves recombination or formation of micropores in the membrane [120]. Dialysis, freeze-thaw cycles, electroporation, sonication, surfactant treatment, and extrusion are all methods used to actively load drugs [210]. These methods enhance MEV loading efficiency but damage the MEV membrane, inactivate proteins, promote MEV aggregation, and expose MEVs to immune cells [211].

Passive loading

Passive cargo loading involves the co-incubation of therapeutic agents with MEVs or donor cellular entities (Fig. 6) [219]. Passive methods for drug loading into MEVs are simpler and gentler than active methods, preserving MEV integrity but often resulting in lower encapsulation efficiency [220]. Primary passive methods include incubation, co-incubation during MEV isolation, diffusion, lipophilic interactions, and hydrophobic/hydrophilic

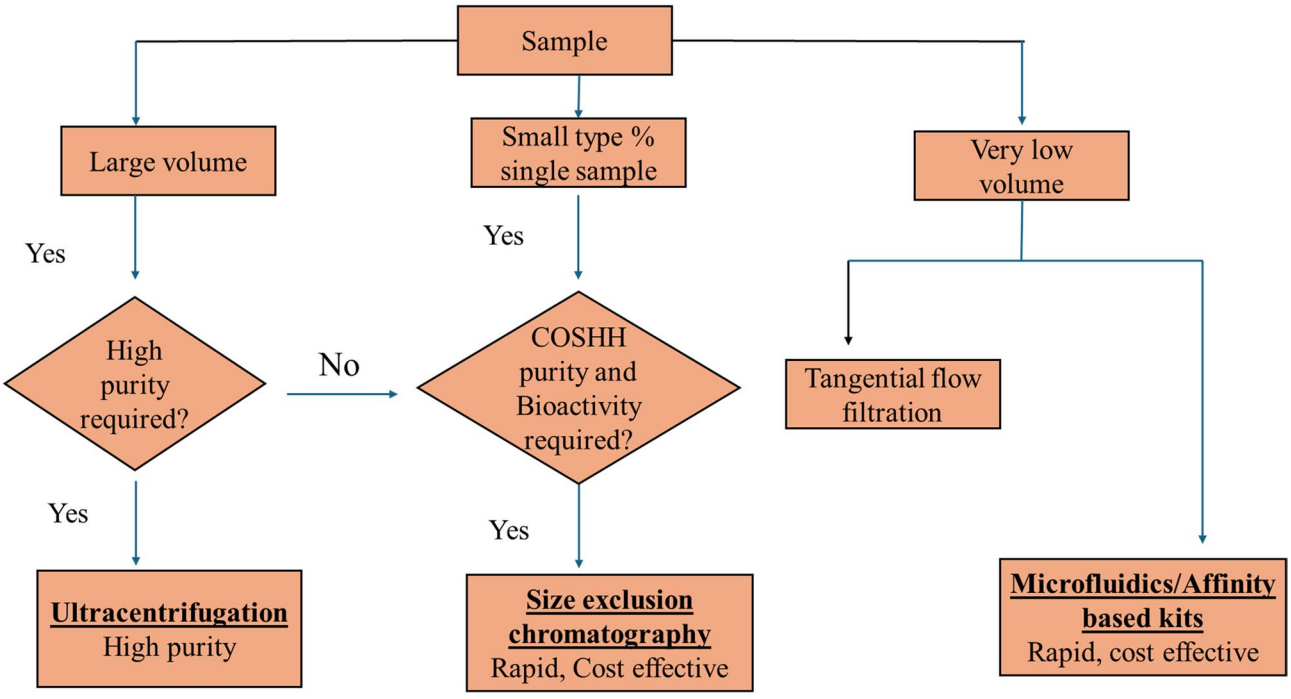


Fig. 5 Flow chart for method selection for isolation of MEVs. This figure presents a structured decision-making framework to guide the selection of the most suitable method for isolating MEVs based on sample characteristics, research objectives, and technical requirements. The flow chart outlines the step-by-step considerations involved in choosing between commonly used isolation strategies such as differential centrifugation, ultracentrifugation, density gradient separation, size-exclusion chromatography, filtration-based methods, and microfluidics platforms

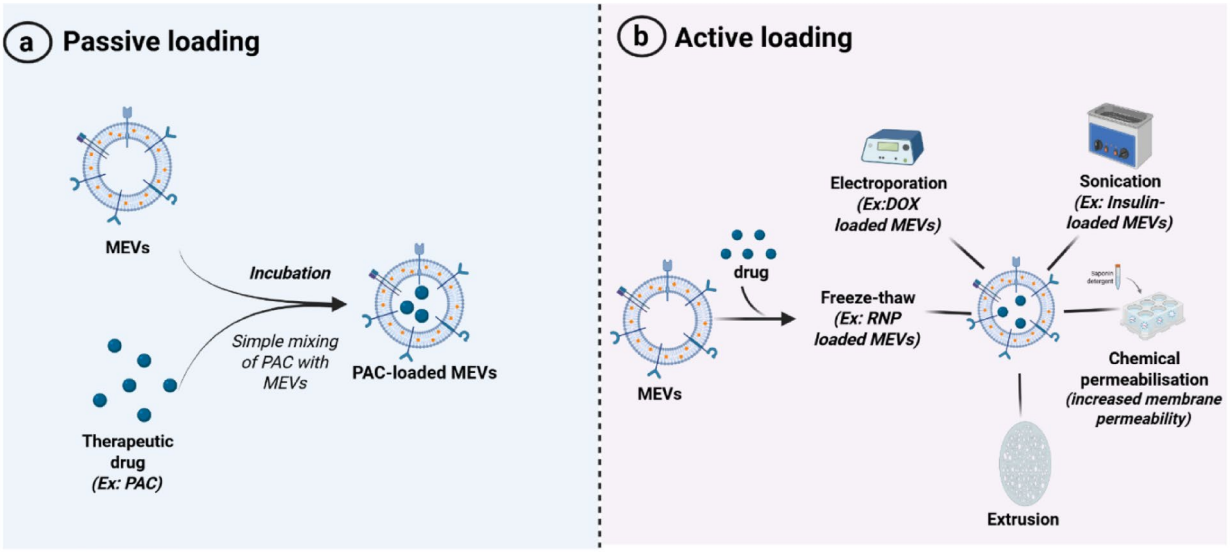


Fig. 6 Strategies for loading therapeutic agents into MEVs Schematic overview of MEV loading approaches, classified into (**a**) passive and (**b**) active techniques. Passive loading involves simple incubation and diffusion, allowing therapeutic cargos to associate with MEVs through the inherent permeability of the vesicle membrane. Active loading methods, including electroporation, sonication, freeze thaw cycling, extrusion, and chemical permeabilization, utilize external physical or chemical stimuli to transiently alter membrane properties and enhance cargo incorporation

partitioning [201]. Incubation involves mixing MEVs with the drug and incubating them under optimal conditions, whereas co-incubation introduces the drug during MEV isolation. Agrawal AK et al.. developed a modified MEV for oral delivery of chemotherapeutic drug paclitaxel (PAC) by loading drug by simple mixing of PAC solution to overcome toxicities and improve patient compliance. ExoPAC showed stability, tumor growth inhibition, and lower toxicities compared to i.p. PAC aims to improve patient compliance [163]. Diffusion relies on the

concentration gradient to drive drug molecules into the MEV membrane or interior, whereas lipophilic interactions use the natural affinity of lipophilic drugs to embed into the lipid bilayer of MEVs. Hydrophobic/hydrophilic partitioning adjusts the solvent conditions to facilitate drug partitioning into the hydrophobic core or aqueous interior of the MEVs [219]. Passive methods generally yield a lower loading efficiency than active techniques, but they are gentler and preserve MEV structure and functionality [221]. They are best suited for small molecules, particularly hydrophobic drugs, that naturally interact with the lipid bilayer of vesicles. However, they are not ideal for certain drugs, such as large molecules or hydrophilic compounds, and may require post-loading purification to remove the free, unencapsulated drugs. The drug diffuses into the MEVs along its concentration gradient; however, this method risks loading efficiency, as hydrophobic drug molecules may traverse both the MEVs and host bilayer membrane [30].

Active loading

Active methods for drug loading into MEVs aim to enhance the drug encapsulation efficiency and ensure functional integration (Fig. 6). These methods use physical, chemical, or biological strategies to promote drug loading [221]. Common active methods include electroporation, freeze-thaw cycles, sonication, extrusion, chemical permeabilization, covalent or non-covalent conjugation, hypotonic dialysis, ultrasound, and microfluidic devices [222]. Electroporation disrupts the vesicle membrane using an electric field, allowing drugs to enter but causing aggregation or vesicle damage if not optimized [223]. Mukhopadhya A et al. found electroporation to be a more effective method for loading Dox into MSC EVs and MEVs despite sonication exhibiting fewer $CD9^+$ EVs/mL and $CD63^+$ EVs/mL, suggesting that it may have negative effects [224]. Sonication creates temporary pores in the vesicle membrane, facilitating the entry of both hydrophobic and hydrophilic drugs. Wu L et al.. found that insulin-loaded MEVs (EXO@INS) demonstrated better hypoglycemic effects than subcutaneously injected insulin, with a sonication method achieving 57.7% and 15.9% drug loading efficiency [225]. Ultrasound is a highly effective method for modifying the size and form of MEVs, enabling their efficient absorption by target cells. It ensures high drug loading efficiency and sustained release, but does not induce nucleic acid aggregation, which is important for MEV aggregation [222, 226]. Extrusion forces EVs and drugs through defined pore sizes, thereby ensuring physical interactions and drug encapsulation [227]. Zhang et al.. successfully loaded PAC into EVs from human umbilical cord-derived mesenchymal stem cells (huc-MSCs) using a lipid extruder. The loading efficiency was 14.23 ± 0.25%, higher than conventional freeze-thaw treatment. Drug-loaded EVs successfully induced neural stem cell differentiation into neurons, demonstrating the effectiveness of this approach [228]. Freeze-thaw cycles freeze and thaw EVs with the drug, causing membrane disruption and facilitating drug loading. Zhuang et al. used a freeze-thaw cycling method for loading RNP into MEVs, that resulted in a loading efficiency of 37.62% compared to ultrasonication [229]. Chemical permeabilization temporarily increases membrane permeability but can lead to residual chemicals or vesicle damage. Covalent or non-covalent conjugation attaches drugs to the MEV surface or within via chemical linkers or affinity-based interactions but can be complex and expensive [222]. Hypotonic dialysis creates an osmotic gradient to swell MEVs, increasing membrane permeability for drug entry but is limited to hydrophilic drugs [230]. Microfluidic devices mix MEVs and drugs under controlled conditions to enhance encapsulation efficiency, but require specialized equipment [231]. Loading efficiencies vary among drug types; however, the highest efficiencies are often achieved by sonication, extrusion, and saponin detergent permeabilization [232]. The main disadvantage of the active loading strategy is that it weakens the structural integrity of MEVs and reduces the number of proteins on their surfaces, thus preventing them from functioning [233].

Quantification of MEVs

MEVs characterization is essential for determining the MEV quantity, identifying their locations, and evaluating how non-EV components contribute to EV preparedness [234]. The particle size, chemical heterogeneity, changing sizes, lack of tools for universal EV authentication, and non-EV specificity of many measuring techniques hinder characterization [235]. Therefore, no single measurement or technique can fulfil all the MEVs characterization requirements [236]. Characterization methods include quantifying particle number concentration, particle size, total protein content, total lipids, total RNA, and EV morphology.

Quantification of particle number concentration

MEV population and volume measurements may be used to calculate the amount (in particles/mL), a frequently reported metric used for assay output measurements, assay input standardization, and in vivo dosing [236]. The ISEV Rigor and Standardization EV Reference Material Task Force highlighted the need for testing LODs and challenges associated with EV concentration detection [237]. Orthogonal approaches with well-established limits of detection (LODs), such as resistive pulse sensing (RPS) technologies and optical techniques, such as flow cytometry, can boost confidence in the estimation of MEV concentrations [238]. Membrane dyes can cause self-aggregation, complicating the differentiation

between MEVs and other co-isolates and potentially leading to an overestimation of MEVs [236]. If these techniques are unable to distinguish MEVs from other possible co-isolates or suspended pollutants, it is advised that the concentrations for upstream separation operations be reported as "particle or EP concentration" rather than "MEV concentration" [239].

Particle size quantification

MEV size measurements rely on assumptions regarding sphericity and mobility, which affect MEV production [240]. Although high-throughput methods, such as DLS, NTA, flow cytometry, RPS, and multi-angle light scattering assume spherical MEVs, the origins of these notions may lead to differences in the measurement methods used [241]. Techniques for determining the particle diameter that rely on proprietary algorithms could differ depending on the platform or program version. When reporting a diameter, methods that are unable to differentiate EVs from co-isolates or pollutants should use the terms "particle" or "EP" diameter [236].

Quantification of the total protein content

The total protein content in an MEV preparation can be ascertained using various methods, including colorimetric, fluorometric, and global protein staining on SDS-PAGE, and spectrophotometry [242]. However, because of co-isolated proteins, total protein measurement frequently overestimates MEV content, particularly for less precise techniques or complicated biofluids [236]. Low-yield and high-purity MEV preparations can cause problems with test sensitivity (265). Information on physical disruption and detergent concentration should be included, as protein concentration can vary depending on whether MEVs are intact or disrupted [244].

Quantification of total lipids

Various analytical techniques can be employed to ascertain the total lipid content in MEV samples. These methodologies include colorimetric assays [245], fluorescence using membrane-intercalating dyes, FTIR, and chromatographic approaches [246]. However, FTIR and intercalating dye techniques are not sufficiently sensitive for trace levels of MEVs, and certain techniques require extremely specialized machinery [247]. Total lipid tests may exaggerate MEVs because of the presence of lipoproteins and other co-isolated NVEPs [248].

Quantification of total RNA

The quantification of RNA, a chemical linked to EVs that has been extensively studied, is essential for quality control and normalization in functional investigations and profiling [249]. Capillary electrophoresis and other methods can measure total MEV RNA [250]. Isolation kits can affect results, and some RNA quantification methods cannot differentiate between RNA and DNA. Many dyes fail to distinguish between DNA and RNA, necessitating specific bioanalyzer kits for short RNAs. Although RNase-free DNase pretreatment may not fully remove DNA contamination, it can improve RNA measurement accuracy [251].

Characterization techniques used to determine the MEV morphology

The most effective methods for evaluating MEV morphology are high-resolution imaging techniques (Table 3), such as cryo-EM, atomic force microscopy (AFM), scanning probe microscopy (SPM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) [252, 253]. Conventional light microscopy can also be applied to larger MEVs. These methods may not be interchangeable or can produce images of the same level of quality. For example, EVs may produce an artifactual cup shape in dehydrated settings, which is not observable under hydrated conditions. If imaging techniques can effectively visualize co-isolated non-vesicular extracellular particles (NVEPs), they could facilitate the particle-level assessment of MEV purity [254].

Bead-based flow cytometry

Flow cytometry using beads is a popular technique for studying MEV surface proteins [255]. Regardless of the makeup of the particles, large beads or beads coupled with antibodies can collect particles and reveal associated antigens [256]. An affinity reagent that is fluorescently attached was used to identify bead-associated particles for easier detection. There are semi-quantitative variations in the staining intensity, as each bead records the signals of several particles. To validate signal titration and mitigate nonspecific binding, control protocols should incorporate isotype-matched detection antibodies, capture beads exclusively containing the detection antibody, and a range of MEV input quantities [257].

Single-EV flow cytometry

Flow cytometry is an optical technique that utilizes light scattering and fluorescence to differentiate between vesicles as small as 40 nm under certain circumstances and 100 nm in conventional contemporary cytometers [258]. Particle diameter, epitope density, epitope abundance, effective refractive index, and concentration were characterized within a specific size range [237]. The accuracy and consistency of single-EV flow cytometry findings are largely dependent on light scattering and fluorescence parameter calibrations [205]. Image cytometers encounter challenges in defining and standardizing the lower detection limit when using a dynamic triggering technique [259].

Table 3 Characterization of MEVs categories, methods, principles and significance

Category of characterization	Method	Principle	Significance	Reference
Particle number concentration	Dynamic Light Scattering (DLS)	• Utilizes Brownian motion of particles • Causes fluctuations in scattered light intensity • Results in constructive and destructive interference patterns • Uses autocorrelation function for particle hydrodynamic radius calculation	• Measures the hydrodynamic size of MEVs • Provides polydispersity assessment • Non-invasive technique without altering structural integrity • Combines with other techniques for comprehensive size distribution and protein content data	[285, 286]
	Nanoparticle Tracking Analysis (NTA)	• Utilizes Brownian motion to track particle movement in fluids • Smaller particles exhibit faster movement • Light scattering occurs at a 90-degree angle to illumination source • Enables visualization and tracking of individual particles	• Provides high-resolution data for understanding MEV population heterogeneity • Integrates fluorescence for identifying specific MEV subpopulations. • Facilitates real-time tracking of MEV dynamics for studying biological functions • Addresses variability issues with different analytical techniques • Significantly impacts clinical diagnostics through accurate characterization and quantification	[273, 287]
	Flow cytometry	• Transports particles suspended in a fluid stream through a laser beam • Aligns particles for individual analysis • Employs fluorescently labelled antibodies for surface marker binding • Particles are excited and emit light at different wavelengths • Detectors capture emitted fluorescence for subpopulation identification and quantification	• Allows simultaneous measurement of multiple parameters • Improves sensitivity and resolution for analyzing smaller EVs • Identifies specific EV subpopulations using surface proteins or biomarkers • Directly applies to biological samples, reducing processing time • Provides a framework for MEV analysis standardization	[288, 289]
	Resistive Pulse sensing (RPS)	• Utilizes a tunable nanopore in an elastic membrane • DC voltage establishes baseline ionic current for particle detection • MEV blocks ionic current flow, causing resistive pulse indicating particle size and concentration • Pulse amplitude relates to particle size, providing statistical data • RPS can assess zeta potential of MEVs	• Offers single-particle MEV analysis via nanopore • Determines particle diameter through linear relationship between MEV volume and resistive pulse • Quantifies MEV size and concentration based on blockade event frequency • Assesses zeta potential through pulse duration analysis	[290, 291]
Particle size quantification	Transmission Electron microscopy (TEM)	• Utilizes a focused beam of electrons to create high-resolution images of internal and external structures in materials • Interactions occur between electrons and the sample's atomic structure, with regions with higher atomic numbers scattering more electrons • Electromagnetic lenses focus transmitted electrons, forming a final image with a resolution of up to 0.1 nm	• TEM provides high-resolution observation of individual MEVs and their structural details • It provides morphological insights into MEVs' size, shape, and surface characteristics • TEM allows for content and purity evaluation, important for therapeutic applications • When combined with immunogold labelling, TEM can detect and localize specific proteins on MEVs surfaces	[292]
	Scanning Electron microscopy (SEM)	• Uses a focused beam of high-energy electrons to create detailed images of a sample's surface • Involves generating a high-energy electron beam, scanning the sample in a raster pattern, and detecting emitted signals • Signals include secondary electrons, backscattered electrons, characteristic X-rays, auger electrons, and cathodoluminescence • Detectors convert these signals into electrical signals, forming a high-resolution image reflecting the sample's properties	• Offers high-resolution imaging for differentiation between EV types • Provides comprehensive information about MEVs like size, shape, and surface texture • Offers sample preparation flexibility for larger MEV population • Enhances image quality and throughput while preserving MEV morphology	[293]
	Other methods include NTA, DLS, RPS			

Table 3 (continued)

Category of characterization	Method	Principle	Significance	Reference
EV morphology	Methods include Electron Microscopy such as TEM, SEM, NTA, Flow cytometry			
Quantification of the total protein content	Bicinchoninic Acid (BCA) Assay	• The BCA assay is a colorimetric method that quantifies protein concentration based on the reduction of Cu^{2+} to Cu^{1+} by proteins in an alkaline environment, forming a coloured complex with BCA reagent	• Quantifies total protein content in MEVs • High sensitivity for detecting low protein concentrations • Compatible with various buffers and diluents for accurate MEV isolation • Quick, straightforward, and requires minimal sample preparation	[294]
	Qubit Fluorometric Assay	• Utilizes fluorophore-based dyes to bind to specific biomolecules • Shows minimal fluorescence in unbound state, but produces strong fluorescent signal upon binding • Allows precise quantification of biomolecules in a sample	• Utilizes fluorescent dyes for high sensitivity and specificity • Detects protein concentrations as low as 12.5 µg/mL • Provides quick quantification results in high-throughput settings • Ensures accurate quantification in complex biological mixtures • Available for double-stranded DNA, RNA, and proteins	[295]
	NanoOrange Assay	• The NanoOrange Assay quantifies proteins in solution using fluorescence, detecting protein concentration through the interaction of NanoOrange, a fluorescent dye, with proteins, resulting in a significant increase in fluorescence	• High sensitivity for accurate measurements from limited sample volumes • Rapid, efficient protocol for high-throughput screening • Requires minimal sample preparation, reducing loss and degradation risk • Importance in MEV research for accurate protein content understanding	[296]
	Bradford Assay	• The colorimetric assay measures protein concentration by binding Coomassie Brilliant Blue dye to proteins, resulting in a colour change that can be measured spectrophotometrically	• Quantifies total protein content in biological samples • Simple, fast, and sensitive to low protein concentrations • Provides insights into MEV concentration • Used as a quality control measure for MEV preparations	[235]
	Western blot	• Detects and analyzes specific proteins in biological samples • It combines gel electrophoresis with immunoblotting to achieve both separation and identification of proteins • Uses gel electrophoresis to separate proteins based on molecular weight	• Important for analyzing MEVs • Identifies and characterizes protein markers associated with MEVs • Helps understand MEVs roles in inflammation, cancer progression, and immune responses • Serves as a quality control measure during MEV isolation protocols • Changes in MEV protein composition can serve as biomarkers for diseases	[297]

Table 3 (continued)

Category of characterization	Method	Principle	Significance	Reference
Quantification of total lipids	Sulfo-Phospho-Vanillin (SPV) Assay	• The SPV assay is a colorimetric method that quantifies lipids based on the reaction of phospholipids with sulfo-phospho-vanillin reagent, resulting in a colored complex that can be measured spectrophotometrically	• Composition influences MEVs structural integrity and functionality • Understanding lipid composition aids in MEVs interaction with the environment and biogenesis • Variations in lipid levels affect MEV production and release mechanisms • Lipid profile changes identify biomarkers for disease diagnosis	[298]
	Förster Resonance Energy Transfer (MS) Assay	• The FRET method quantifies the total lipid membrane surface area in a sample by causing fusion between vesicles and liposomes containing fluorophores, with the emission spectrum change corresponding to the total membrane surface area	• Quantifying Total Lipid Membrane Surface of MEVs • Essential for understanding biophysical properties and cell interactions • Capable of quantifying MEVs • Sensitivity and low sample volume requirements • Independent of vesicle size, allowing accurate quantification • Simple, robust methodology, accessible for routine use in MEV labs	[299]
	Mass Spectrometry (MS)	• Mass spectrometry provides detailed lipidomic analysis by measuring the mass-to-charge ratio of lipid molecules allowing for comprehensive profiling of lipid species within MEVs	• Important for lipid content analysis • Can detect over 260 lipid molecules • Identifies novel lipid biomarkers linked to diseases • Profiles MEV lipid composition from various cell types • Uncovers specific lipid signatures as diagnostic or prognostic markers • Provides insights into MEVs role in intercellular communication and disease progression	[300]
	Infrared (IR) Spectroscopy	• FTIR spectroscopy is a powerful analytical technique used to examine the interaction between infrared radiation and molecular vibrations within lipid molecules in MEVs • Identifies and analyses chemical compounds • Relies on the absorption of IR radiation • Causes vibrational and rotational transitions within molecules	• Important tool for MEV lipid content analysis • Provides comprehensive lipid profiling based on characteristic absorption peaks • Non-invasive nature allows quick analysis of MEVs • Calculates protein-to-lipid ratios for MEV composition and purity insights • Reveals dynamic changes in lipid composition and organization • Allows simultaneous assessment of lipids alongside other biomolecules	[301]
Quantification of total RNA	SMARTer Stranded Total RNA-Seq Method	• Quantifies total RNA from samples using SMART technology • Converts total RNA into complementary DNA using specific probes • Prepares library through adapter ligation and amplification • Ensures quality control and sequencing through purification steps • Analyses libraries using Illumina sequencing technologies for comprehensive RNA species profiling	• Advances RNA sequencing for human biofluid analysis • Offers low input requirements, transcriptome profiling, strand-specific sequencing, and ribosomal RNA depletion • Works efficiently with minimal RNA quantities • Preserves RNA strand orientation for accurate gene expression quantification	[302]
	Spin Column-Based Isolation	• Combines centrifugation and solid-phase extraction for high purity	• High purity and yield • Ensures RNA extraction primarily from MEVs • Can handle up to 4 mL sample volumes • Faster than ultracentrifugation	[249]
	NanoString nCounter Platform	• Utilizes digital fluorescent barcode technology for direct RNA measurement • Uses capture probes tagged with unique barcodes for precise identification and quantification • Minimizes biases during cDNA synthesis for accurate quantification • Requires as little as 1 ng of RNA input per sample	• High reproducibility across experiments • Rapid turnaround time • Comprehensive data analysis • Digital counting mechanism for longitudinal studies • Streamlined workflow within 24 h	[303]

Atomic force microscopy

Atomic force microscopy (AFM) is one method that offers label- and stain-free imaging of single EVs and co-isolated nanoparticles [260]. To perform measurements, analytes must be set up on a solid surface, after which they can be dried or immersed in a liquid. AFM morphometry provides data on the EV size distribution, ultrastructural features, and contamination status [261, 262]. MEVs can be separated from similarly sized and shaped NVEPs using their unique mechanical fingerprints [263]. Imaging MEV samples with AFM requires detailed reporting of the initial sample deposition technique, substrate type, preliminary procedures, immobilization methods, sample concentration and deposition times, rinsing and/or drying steps, AFM visualization mode, acquisition conditions, and probe information [264].

Diffraction limited fluorescence microscopy

Cell-MEV interactions, encompassing the release and absorption of MEVs, have been analyzed using fluorescence techniques such as confocal, light-sheet, and total internal reflection microscopy (TIRF-M) [265]. TIRF microscopy is advantageous for single-molecule detection because of its high signal-to-noise ratio and restricted imaging capability at the glass interface [266]. Although confocal and light-sheet microscopes are better suited for live cell imaging tasks, they are capable of identifying individual molecules for dynamic research and calibration [265]. Because of the limited number of labelled molecules in fluorescent samples, it is important to document the microscope type, magnification, laser power, and exposure time during microscopy studies [267].

Dynamic light scattering

DLS technique may be used to calculate the hydrodynamic size of monodisperse components in diluted aqueous dispersions [106]. DLS calculates the autocorrelation function of the laser light scattered by various particles in the solution to determine the diffusion coefficient of the particles. However, to ascertain the quantitative characteristics of MEVs samples, they should be utilized exclusively as monodisperse size fractions [236].

Electron microscopy

Although electron microscopy (EM) is a high-resolution method that can identify MEVs of any size, larger MEVs are statistically underestimated owing to their throughput [268]. SEM, TEM, and cryo-EM are MEV characterization techniques; however, they are not interchangeable and can provide images of similar quality [269]. Compared to TEM, which uses dehydrating conditions to preserve MEV morphology, cryo-EM preserves MEV morphology better and may be more quantitative [270]. To maintain the lipid bilayer structure, TEM must utilize an MEV-specific technique involving uranyl/methylcellulose contrast and embedding [271]. Any size of MEV can be seen from its surface using SEM; however, pictures acquired at maximum magnification may be more challenging to interpret [272].

Nanoparticle tracking analysis

Nanoparticle tracking (NTA) is a widely used optical method in the MEV sector to determine the effective refractive index, verify epitopes, and measure particle size and concentration. Evaluating the diffusion coefficient of a particle with a technique that minimizes the diameter distribution variance allows the determination of the hydrodynamic diameter [273]. MEV-containing particles can be identified by light scattering or fluorescence, which is influenced by the removal of unbound labels, dye photobleaching resistance, and measurable dye presence in each particle [274]. NTA reporting includes the equipment model, camera settings and type, laser strength, wavelength, software version, analysis parameters, and particles per frame [275]. Single-diameter statistics may produce skewed findings and NTA diameter distributions are preferable.

Resistive pulse sensing

Resistive pulse sensing (RPS) is a non-optical technique that measures the concentration, particle diameter, and zeta potential using the Coulter principle [276]. Current implementations feature pre-calibrated fixed pores in microfluidic cartridges and uncalibrated stretchable holes with detection limits as low as ~50 nm in diameter [277]. Large protein complexes and lipoproteins, though often co-isolated, should be cautiously included in RPS assays despite their high concordance with results and TEM data [278]. Reporting RPS findings requires details of the equipment model, pore size, calibration bead diameter, source, software version, applied voltage, stretch, optimization process, and proper dilution buffer for microfluidic RPS [278, 279]. For MEV data, presenting RPS diameter distributions is preferred over a single diameter statistic because of the potential LOD distortion [236].

Western blotting

Western blotting is a method used to detect and identify proteins in fluids containing MEVs. Proteins were separated by gel electrophoresis [280], transferred onto a membrane, and analyzed with affinity reagents, typically antibodies [281]. The MEV preparation or source standardizes the input, enabling the comparison of MEV cargo quantities between groups and assessment of general variations in MEV production and uptake balance

[236]. Cell lysates from cell culture EVs were added to the gel to assess MEV enrichment or depletion relative to cell production [282]. Nevertheless, only EV analysis from cell-culture conditioned media can be used for this comparison [283]. Control samples are essential alongside experimental samples to assess sample purity, especially when claiming the presence of proteins in or on MEVs [284].

Surface modification of MEVs

MEVs can be surface-modified to improve their therapeutic effectiveness and targeting capacity (Fig. 7) [304]. They can be functionalized with exogenous imaging and targeting moieties to offer target selectivity and real-time tracking for therapeutic and diagnostic applications [305]. This strategy enhances selective targeting and drug delivery by modifying the MEV surface with ligands, such as aptamers, sugars, peptides, vitamins, and antibodies [306]. Chemical alterations and genetic engineering enhance the MEV-targeting capabilities and therapeutic index of MEVs [140]. Recently, there has been considerable interest in functionalization of MEVs to provide a range of capabilities for biomedical applications [308]. Three primary approaches have been identified for MEV surface functionalization: chemical, biological, and physical [309].

Physical method

Physical methods, such as extrusion, freeze-thaw, and sonication, have been employed to functionalize MEVs (Fig. 7) [222]. These methods alter surface properties by rearranging the membranes [310]. Physical surface modification of MEVs can potentially improve their functionality for therapeutic applications [176]. These modifications include improved stability, enhanced drug delivery capabilities, and facilitation of intercellular communication [311]. Zhang R et al.. have developed a method for functionalizing BMEVs with nanobody, resulting in an EGFR-targeted drug delivery system. The diglycine-containing amphiphile molecule on the BMEVs membrane was ligated to the EGFR nanobody (7D12), which was loaded with doxorubicin (Dox) and delivered Dox to cancer cells in response to EGFR expression [312]. Another study by Javier et al. found that gadolinium-modified EVs (EVs-GdL) can accumulate within tumours, potentially affecting the tumour location or drug delivery. The study showed that EVs-GdL improved tumour contrast in ectopic tumour-bearing mice with osteosarcoma and showed longer residence time and biological activity. They also compared MEVs labelled with near-infrared dye (DiR) and PEGylated NPs labelled with DiR, revealing their predominant accumulation in the liver. This study suggests that Human Umbilical Cord Mesenchymal Stem Cells (HUC-MSCs) are a potential therapeutic target. Figure 8 shows the distribution of gadolinium-labelled EVs in tumor-bearing mice and near-infrared dye-labelled EVs in mice with ectopic osteosarcoma tumors using magnetic resonance imaging (MRI) [313].

Chemical method and covalent modification

Chemical surface modifications enhance MEVs targeting, stability, and cargo loading by attaching specific ligands or antibodies to their surface (Fig. 7) [314]. They also protect MEVs and their cargo from degradation, especially sensitive cargo, such as RNA or proteins [315]. Chemical modifications also enhance biocompatibility and safety by reducing immunogenicity and ensuring compatibility with human cells [316]. These alterations can influence

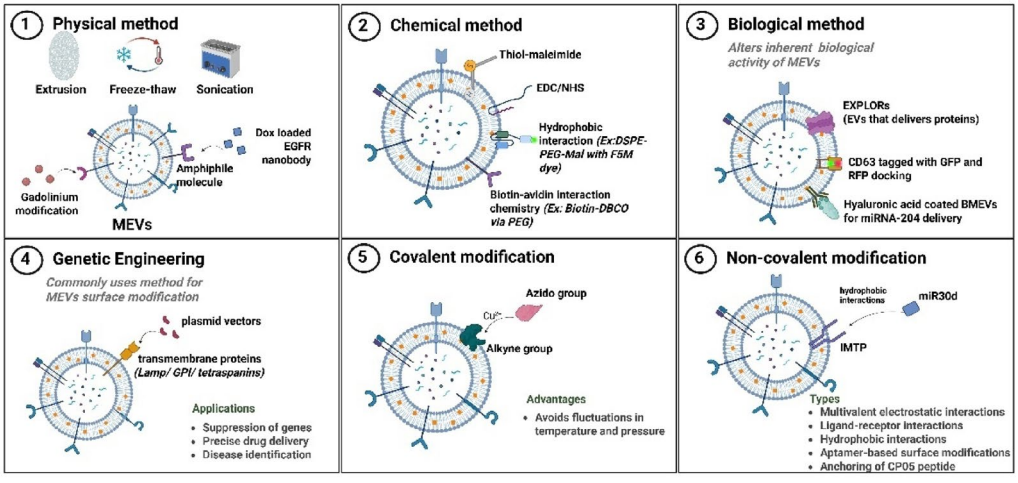


Fig. 7 Strategies for surface modifications of MEVs. This figure illustrates the diverse surface-engineering approaches used to functionalize MEVs for improved therapeutic performance. Surface modification plays a crucial role in enhancing vesicle stability, prolonging systemic circulation, enabling targeted delivery, and modulating immunological interactions. The schematic highlights several key strategies, including chemical conjugation, where ligands, antibodies, peptides, or polymers are covalently attached to MEV membranes to achieve receptor-specific targeting or increased biocompatibility

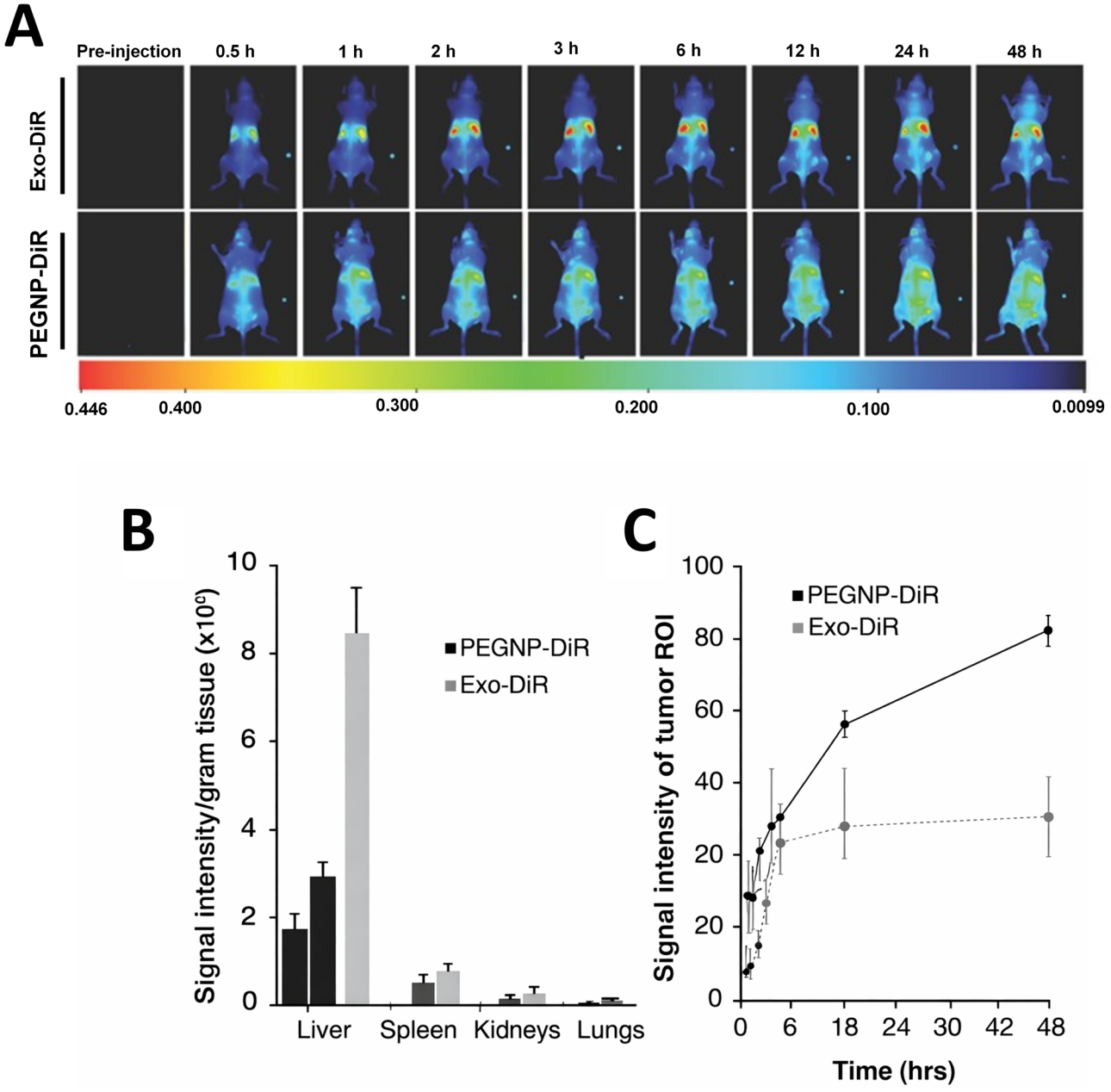


Fig. 8 In Vivo distribution and fluorescence tracking of EV-DiR in tumor-bearing mice. **(A)** Distribution of near-infrared dye labelled EVs (EV-DiR) in tumor-bearing mice. The rapid onset of DiR labelling in PEGNP DiR mice waned over time (**B**) Signal intensity of the organs were assessed (**c**) Fluorescent signal measured at various time points at Tumor region of interest (ROI) was adapted from [261] licensed under CC BY-NC

the biological activity of MEVs, potentially affecting their effects on cellular processes such as inflammation, immune responses, and tissue repair [58]. This chemical method provides simple click-type chemistry to covalently link MEV proteins or lipid structures with various linker groups for diverse functionalities. Amidation, thiol-maleimide, EDC/NHS, and azide-alkyne cycloaddition chemistry have been explored for MEV functionalization [317]. By coupling biotin with DBCO via PEG, Wang et al.. developed biotin functionalization in EVs. Biotin-avidin interaction chemistry enhances the functionalities of EVs, allowing for biomedical applications. Biotinylated EVs can deliver proteins and link cargo, whereas azide functionality expands biotinylation functions. A study revealed that metabolic engineering improves the chemistry and function of EVs, enhancing their biomedical potential [318]. Using Maleimide (Mal) terminated lipids, Di et al. developed a clickable lipid probe method for the separation and enrichment of EVs. This technique conjugates maleimide-EVs with

dithiol-containing molecules, a thiol-containing magnetic particle, and thiol-containing groups, enabling the vibration detection of EV surface cargoes. They have developed a high-efficiency method for EV engineering that allows Mal moieties to conjugate with functional tags for biomedical applications without time-consuming procedures or expensive instruments. This universal technique can be used to engineer various EVs without affecting their morphology or biological properties. (Fig. 7) illustrates the engineering of EVs using a hydrophobic insertion strategy with DSPE-PEG-Mal, labelling them with fluorescent F5M dye, enriching them with MPs, and detecting them using Raman spectroscopy [319].

Click chemistry has been used to modify the surface of MEVs by attaching an alkyne group to them via a condensation reaction and covalently conjugating it to the azido group of the targeting moiety in the presence of copper (Fig. 7) [160]. Compared with traditional chemical reactions, this approach has numerous advantages. It avoids potential issues, such as fluctuations in temperature, pressure, or osmotic pressure, that can result from unsuitable salt concentrations. Click chemistry reactions are highly efficient, and proceed rapidly in both organic solvents and aqueous buffers. The conjugation process did not alter EV size or its absorption by the target cell, indicating that the reaction conditions were suitable for modifying the MEV surface [321]. The metabolic engineering of parent cells is a strategy for producing MEVs using click chemistry [322]. Wang et al.. developed a metabolic engineering and labelling strategy for surface functionalization of EVs using cell metabolic processes. They used residue-specific protein labelling and metabolic glycan labelling to incorporate non-canonical amino acids in newly synthesized proteins, such as L-azidohomoalanine (AHA), which can be expressed in EVs through biogenesis. Metabolic labelling of the azide moiety in secreted EVs was confirmed using Cy3 conjugated dibenzobicyclooctyne (DBCO -Cy3), confirming the successful strategy. The functionalized azide moiety on the EV surface offers opportunities for various azide-based chemistry [318]. Using protein ligases, Pham TC et al.. developed a novel technique for covalently conjugating natural EVs with large copy numbers of targeted moieties. This allows for the accumulation of EVs in EGFR-positive cancer cells both in vitro and in vivo. The method also applies to conjugating EVs with peptides and nanobodies targeting other receptors, such as HER2 and SIRP alpha. Conjugated EVs can deliver RNA and small molecules, supporting their versatile applications in cancer therapies [323]. Modifying MEVs with altered proteins, lipids, or glycans involves the addition of synthetically modified amino acids, lipids, glycans, or oligonucleotides to the culture medium of MEV-producing cells. However, the use of click chemistry for MEV surface modification is constrained by the non-specific binding of the targeting moiety to the MEV surface [324].

Biological method and genetic engineering

The biological approach outlines methods for modifying cellular genes and metabolism to produce specific cargo molecules or proteins in the released MEVs (Fig. 7) [325]. These modifications can incorporate ligands that recognize specific receptors on target cells, facilitating efficient delivery of therapeutic agents [326]. They can also alter the inherent biological activity of MEVs by enhancing or suppressing their anti-inflammatory, tissue-regenerative, and immunomodulatory properties. Yim et al.. developed EXPLORs, an optogenetically designed EV system that delivers proteins intracellularly via surface functionalization. Cell-derived EVs represent an intriguing approach for illness treatment in humans. By integrating a reversible protein-protein interaction module controlled by blue light,

cargo proteins can be successfully loaded into EVs. Treatment with protein-loaded EXPLORs significantly increases intracellular cargo protein levels and function in recipient cells, indicating the potential of EXPLORs as a mechanism for the efficient intracellular transfer of protein-based therapeutics [327]. The new EV surface display technology developed by Zachary et al.. is essential for targeted medication administration and treatment because it promotes cell-cell communication. The study identified a native surface protein, tetraspanins CD9, CD63, and CD81, MP as the anchoring scaffold for nanovesicle EVs. They developed fluorescent reporters for both inner and outer displays on EVs, demonstrated successful display via gene transfection and fluorescence monitoring, and validated the system by demonstrating the intracellular partitioning and secretion of EVs from human HEK293 cells. They focused on the multitransmembrane configuration of CD63 and identified two candidate sites for displaying fusion proteins. The study concluded that the system can continuously produce, secrete, and uptake EVs with minimal effects on normal cell biology. Figure 9 shows the surface display of EVs using the CD63 molecular scaffold, docking RFP to the outer vesicular space and GFP to the inner vesicular space, and analyzing the colocalization of the displayed RFP and GFP in HEK293 cells 72 h after incubation [328].

Coating MEVs enhances their stability and interaction with biological systems [11], improves their resistance to GIT degradation, and facilitates the delivery of proteins, lipids, and nucleic acids [329]. Different coating materials, such as Polyethylene Glycol (PEG), Chitosan, PLGA, Albumin can influence the surface properties of MEVs, thereby affecting their affinity for intestinal cells and absorption [330]. Li D et al. have developed a method for intracellular delivery of miRNA-204 using Hyaluronic

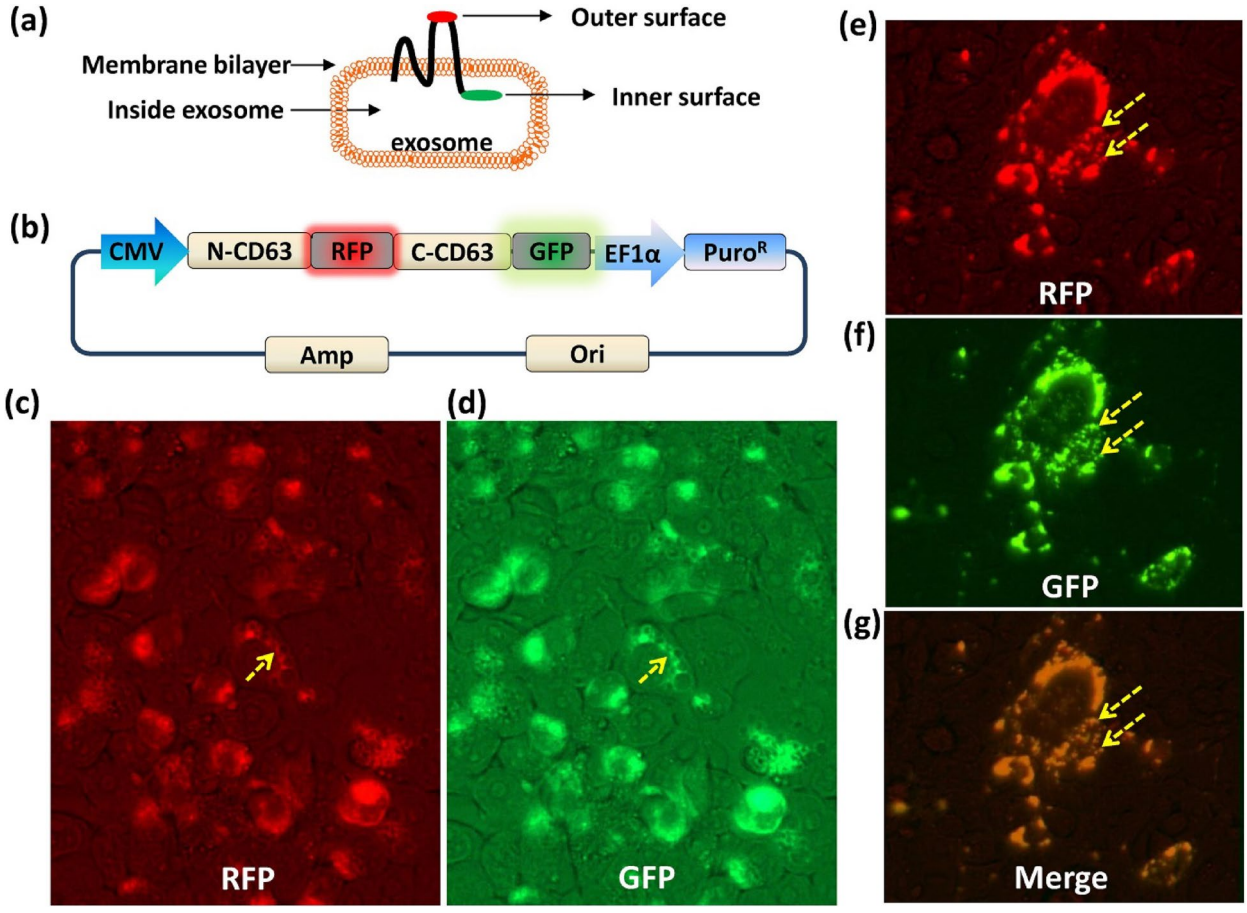


Fig. 9 Surface display of EVs using the molecular scaffold of CD63. (**a**) CD63 scaffold schematic docking of RFP to the outer vesicular space and GFP to the inner vesicular space; (**b**) CD63 fusion protein DNA construct with dual fluorescent markers; (**c,d**) localization of fluorescent markers in HEK293 cells; and (**e,f, g**) colocalization of displayed RFP and GFP in the merged figure in HEK293 cells 72 h after incubation adapted from [272] with license CC BY

Acid-Coated BMEVs, demonstrating increased antitumor efficacy in vitro and in vivo against CD44-positive cancer cells, and targeting the cells through increased intracellular uptake of miR-204 [331]. MEVs demonstrate resilience to adverse conditions within the GIT, including acidic environments and enzymatic degradation [20]. Certain surface proteins on EVs enhance their binding and uptake by intestinal epithelial cells, facilitating targeted delivery, improving cellular communication, and promoting efficient absorption of therapeutic cargo.

Genetic engineering techniques can enhance MEV properties for therapeutic and diagnostic applications (Fig. 7) [156]. A commonly employed method for creating surface-modified MEVs (SMEVs) involves the genetic modification of MEV-producing cells. This process involves the use of plasmid vectors encoding a fusion protein with a targeting ligand and a specific transmembrane protein to enhance the MEV membrane, which is composed of transmembrane proteins such as Lamp, GPI, and tetraspanins [234, 333]. Key strategies include cargo loading, nucleic acid overexpression, protein expression, surface modification, targeting ligands, receptor modification, immune modulation, and MEV production enhancement [334]. Therapeutic applications encompass various areas, including suppression of specific genes, precise drug delivery, immune system-based treatments, early stage disease identification, and the field of regenerative medicine. According to Di et al.., fusion with the transmembrane protein CD47 allows the cancer cell-specific peptides CDX and CREKA to be seen on the EV surface. Parent cells were transfected with a plasmid encoding peptide-CD47 fusion, resulting in modified EVs that showed significant accumulation in brain tumours in vivo. This approach could pave the way for more precise and effective genetic modification [319]. Sato et al. developed hybrid EVs using the freeze-thaw method to control and modify their performance. EVs were embedded with a specific membrane protein from genetically modified cells, demonstrating the potential of membrane engineering methods combined with genetic modification techniques. Cellular uptake studies have shown that the interactions between EVs and cells could be modified by altering the lipid composition or properties of exogenous lipids. Membrane engineering offers a new strategy for

developing rationally designed EVs as hybrid nanocarriers for advanced drug delivery systems [335].

Non-covalent modification

Non-covalent modification of MEVs involves altering their surface properties or functional characteristics without creating permanent covalent bonds or preserving their native structure and functionality, which is important for therapeutic, diagnostic, and research applications (Fig. 7) [314]. Non-covalent techniques, such as multivalent electrostatic interactions, ligand-receptor interactions, hydrophobic interactions, aptamer-based surface modification, and anchoring of the CP05 peptide, are used for targeted MEV production [336].

Multivalent electrostatic interactions

Multivalent electrostatic interactions represent a non-covalent approach to enhance the functionality of MEVs without altering their intrinsic properties. These interactions occur between negatively charged membranes and cationic modifiers, facilitating the reversible attachment of functional molecules or cargo to the MEVs surface. This technology uses various materials and methodologies, including cationic polymers, chitosan, poly-L-lysine, DOTAP, positively charged peptides, nanoparticles, polyelectrolyte layers, and controlled surface functionalization, for therapeutic delivery, ligand attachment, imaging, diagnostics, and biointerface engineering [337–339]. Ridolfi et al. examined the adsorption of MEVs onto supported lipid bilayers (SLBs) to elucidate the role of electrostatic interactions. Utilizing a quartz crystal microbalance with dissipation monitoring (QCM-D) and confocal laser scanning microscopy (CLSM), the study observed that electrostatic forces enable irreversible MEV binding to charged lipid membranes while preventing full molecular contact due to residual counterions. These findings indicate that the surface charge influences MEV interactions at the nanoscale, contributing to intercellular communication [340]. Tamura et al.. found that surface functionalization of EVs with pullulan, a cationic polysaccharide polymer, enhanced their accumulation in hepatocellular carcinoma (HepG2) cells in vitro. Furthermore, functionalized EVs show enhanced accumulation and anti-inflammatory effects in vivo in mice with liver injury, demonstrating their efficacy in treating liver injury [341].

Ligand-receptor interactions

Ligand-receptor interactions are essential for the selective targeting and functional delivery of MEVs to specific recipient cells for therapeutic and diagnostic applications [168]. Natural ligand-receptor systems include Integrin-Ligand Interactions, Tetraspanins, Immune Modulators, Receptor-Ligand Pairs, and Engineered Ligand-Receptor Systems [169]. These challenges include receptor availability, off-target effects, and stability [344]. Studies have used natural receptors on EV surfaces to attach targeting ligands. Transferrin-conjugated superparamagnetic nanoparticles were affixed to EVs produced from blood reticulocytes (RTCs) by Qi et al.. EV-based drug delivery vehicles exhibit superparamagnetic behaviour at room temperature and a stronger response to an external magnetic field than individual nanoparticles. This allows EVs to be separated from the blood and target the diseased cells. In vivo studies using murine hepatoma 22 subcutaneous cancer cells showed that drug-loaded EV-based vehicle delivery enhanced cancer targeting under an external magnetic field and suppressed tumor growth [345]. Using a molecular combination of bovine lactoferrin and poly-l-lysine, Shandilya et al. demonstrated a scalable and affordable technique for loading exogenous siRNA into EVs. This method achieves transfection efficiency comparable to that of electroporation and ensures effective siRNA delivery and uptake by recipient cells [346]. These interactions have demonstrated potential as targeting ligands.

Hydrophobic interaction/membrane engineering

Hydrophobic interactions and membrane engineering are essential techniques for improving the functionality, stability, and targeting capability of MEVs. These methods leverage the hydrophobic nature of MEV membranes and their lipid bilayers to incorporate bioactive molecules, enhance drug delivery, and tailor the surface properties [304]. Functionalizing the MEV membrane with a peptide, antibody, or PEG, followed by freeze-thawing, creates a hydrophobic interaction that enables efficient targeting while preserving MEV membrane protein function [335]. Tong et al. developed miR30d-mEVsIMTP, a targeted delivery system for miR30d in cardiac therapy, using an ischemic myocardium-targeting peptide (IMTP) and encapsulated miR30d. This formulation efficiently entered hypoxia-induced H9C2 cells via the endolysosomal pathway. In cardiac hypertrophy mouse models, miR30d-MEVs IMTP showed enhanced accumulation in the heart tissue, alleviated hypertrophy, and improved cardiac function. GRK5 has been identified as a novel miR30d target for hypertrophy. This IMTP-MEV-based platform offers significant potential for advancing gene therapy for heart diseases [347]. By engineering EVs without compromising their biological capabilities, Lee J et al.. have made it possible to effectively and carefully incorporate functional agents such as drugs, lipids, fluorophores, and bio-orthogonal compounds. They used membrane fusogenic liposomes to engineer parental cells while keeping EVs intact. This method was applied to cancer cells by conjugating EVs with targeting peptides using copper-free click chemistry. This liposome-based

cellular engineering method may be useful for studying the biological roles of EVs and delivering therapeutic agents [348].

Aptamer based surface modification

Aptamer-based surface modification is a method for functionalization of MEVs with high specificity and versatility. These DNA or RNA molecules bind to specific targets with a high affinity, making them ideal for targeted delivery, diagnostics, and therapeutic applications [349]. They can be attached to MEVs using various methods, offering stable and robust binding, and maintaining MEV integrity [350]. To examine tumour-derived EVs expressing PD-L1, a important biomarker for immunotherapy response, Le et al.. devised a technique utilizing a dual-aptamer proximity ligation experiment. This method achieved 100% accuracy in distinguishing patients with cancer from healthy donors, demonstrating its potential for early cancer diagnosis and treatment [352]. Hosseini et al.. developed a nanocarrier system called DOX-Apt-EVs loaded with DOX for the treatment of colorectal cancer. The system, which is approximately 200 nm in size, effectively targets cancer cells and suppresses tumour growth in ectopic mouse models. In vivo experiments have shown that AS1411 aptamer-functionalized EVs can be recommended for site-specific drug delivery in clinical applications for colon cancer. The system exhibited a uniform size distribution, biocompatibility, and homing tumor ability, slowing cancer cell proliferation after intraperitoneal administration (382). The limitations of aptamer modification include stability in biological fluids, off-target binding, optimization of loading efficiency, multivalent functionalization, stimuli-responsiveness, and CRISPR/Cas system integration [353].

Modification by anchoring CP05 peptide

The CP05 peptide, which binds to tetraspanin, facilitates the expression of therapeutic moieties on the MEV surfaces, thereby enhancing drug loading through direct fusion with the therapeutic moiety. They preserve MEVs inherent size and physical traits of MEVs without altering their in vivo distribution [174]. This enables the enhancement of the targeting, imaging, and therapeutic functions of MEV-based systems by attaching functional molecules or payloads to EVs [354]. CP05 can be conjugated to functional molecules such as drugs or imaging agents and displayed on the MEV surface. The diverse expression of CD63, payload size restrictions, immunological recognition, and scalability are obstacles that need to be overcome. Gao et al. discovered that a peptide (CP05) could target and capture EVs without altering their surfaces, unlocking their therapeutic potential. This peptide, identified by phage display, enables the loading and capture of EVs from various sources. EVs loaded with CP05-modified dystrophin increased dystrophin protein levels 18-fold in mice. This study demonstrates the potential of EV anchor peptides for EV engineering and targeted drug delivery [355]. A summary of MEV surface modification strategies, including their advantages and limitations, has been provided in Table 4.

Design and application of modified MEVs for targeted and nucleic acid-based drug delivery

Overview of therapeutic potential

MEVs exhibit broad therapeutic potential owing to their natural biocompatibility, stability, and ability to deliver diverse bioactive molecules across biological barriers. These nanoscale vesicles play crucial roles in maintaining physiological homeostasis across multiple organ systems. In neurological contexts, MEVs promote dendritic development, attenuate seizure severity, and enhance spatial learning and memory, highlighting their potential for managing neurodevelopmental and neurodegenerative disorders. Within the gastrointestinal system, they support epithelial proliferation, repair, and migration, attenuate necrotizing enterocolitis, and mitigate inflammation in ulcerative and DSS-induced colitis models. Their miRNA cargo further contributes to immune maturation, metabolic regulation, and gut microbiota modulation. In musculoskeletal health, MEVs enhance skeletal muscle function, promote bone formation, increase bone mineral density, and prevent bone loss in models of osteoporosis, obesity, and ovariectomy, supporting their potential as prophylactic or therapeutic agents. Cardiovascular studies show MEVs reduce cardiac fibrosis, enhance pro-angiogenic factors, and improve cardiac function, indicating a role in cardiac repair. In dermatological applications, MEVs aid tissue repair, mitigate UV- and burn-induced damage, and facilitate scar-free healing. Collectively, these functions position MEVs as multi-functional bioactive agents with both prophylactic and therapeutic relevance [361].

MEVs as natural drug delivery vehicles

Beyond their physiological roles, MEVs function as efficient natural nanocarriers for therapeutic delivery. Their lipid bilayer structure enables encapsulation of small molecules, peptides, proteins, and nucleic acids, offering protection from enzymatic degradation while enhancing stability, bioavailability, and cellular uptake. MEVs are advantageous over synthetic nanocarriers due to their scalability, biocompatibility, low immunogenicity, and ability to cross physiological barriers such as the intestinal epithelium and BBB Media Play Pause. Functionalization of MEVs allows delivery to specific sites in cancer, inflammatory, and infectious diseases. Preclinical studies highlight systemic therapeutic applications, including

Table 4 Summary of MEV surface modification strategies: Advantages and limitations

Modification strategy	Typical methods	Key features/Mechanism	Therapeutic advantages	Limitations/Challenges	Applications	References
Physical Methods	Extrusion, sonication, freeze-thaw	Alters membrane structure, reshuffles lipids	Improves vesicle stability, enhances cargo encapsulation, facilitates intercellular delivery	May cause vesicle aggregation or partial loss of cargo; limited targeting specificity; risk of structural damage	Rapid, simple; suitable for preclinical drug loading and delivery studies	[356]
Chemical/Covalent Methods	Click chemistry, EDC/NHS coupling, biotinylation, maleimide conjugation	Forms stable covalent bonds between surface ligands and MEV proteins/lipids	High specificity targeting; protects sensitive cargo (e.g., RNA, proteins); allows multifunctionalization for imaging, diagnostics, and therapy	Requires chemical expertise; potential non-specific binding; may alter surface properties or uptake; reaction conditions must be carefully controlled	Targeted drug delivery, imaging, theranostics; scalable with optimization	[377, 378]
Biological/Genetic Engineering	Plasmid transfection, EXPLORs, surface display using CD63/CD81/CD9	Modifies parent cells to produce MEVs with desired proteins, ligands, or nucleic acids	Enables precise ligand display and cargo loading during biogenesis; enhances therapeutic and diagnostic efficacy; supports targeted gene/protein delivery	Complex and time-consuming; safety and regulatory concerns; may require extensive optimization for yield; scalability is challenging	Gene therapy, cancer therapy, regenerative medicine, immune modulation	[359]
Non-Covalent/Physical-Biochemical	Electrostatic interactions, hydrophobic insertion, ligand-receptor binding, aptamer conjugation, CP05 peptide anchoring	Reversible binding without permanent chemical modification	Preserves vesicle integrity; flexible and tunable functionalization; minimal chemical alteration; allows post-isolation modification	Lower binding stability than covalent methods; off-target interactions possible; may require repeated dosing; efficiency can vary	Targeted drug delivery, imaging, nucleic acid delivery, tissue-specific targeting	[360]
Surface Coating Methods	PEGylation, chitosan, PLGA, albumin	Forms protective layer around MEVs	Enhances circulation time, protects against GIT degradation, improves cellular uptake	May interfere with natural receptor interactions; can mask functional ligands; potential immunogenicity with repeated dosing	Oral drug delivery, systemic therapy, enhanced bioavailability	[90]
Hybrid/Combined Methods	Membrane fusion, liposome-assisted surface engineering	Combines physical, chemical, and genetic strategies	Allows multifunctional MEVs with improved targeting, cargo loading, and stability	Complexity increases with multiple modifications; regulatory approval may be challenging; risk of altering native vesicle function	Advanced drug delivery systems, targeted therapies, precision medicine	[335]

cancer therapy (breast and lung), metabolic disorders such as diabetes, and infectious diseases, often with improved bioavailability, reduced adverse effects, and the possibility of oral administration of drugs traditionally delivered intravenously [361].

Early studies have validated MEVs' ability to encapsulate and deliver small molecule therapeutics such as paclitaxel and withaferin A, achieving enhanced antitumor efficacy in vivo. More recent work has extended their utility to nucleic acid-based drugs, including siRNAs and gene modulators, achieving controlled gene silencing, modulation of oxidative stress, and accelerated wound healing. These findings underscore the versatility of MEVs as next-generation delivery platforms capable of overcoming the limitations of conventional systems.

MEVs can be efficiently isolated in large quantities from milk, providing a sustainable, scalable, and cost-effective platform for clinical translation. Their inherent stability under certain processing conditions further supports potential commercialization. Compared with alternative

drug delivery systems, MEVs exhibit biocompatibility, non-toxicity, and low immunogenicity, thereby reducing the risk of adverse reactions. Their capacity to traverse biological barriers enhances their potential for delivering therapeutic agents to challenging areas, while extended circulation time improves bioavailability. Collectively, these features position MEVs as multifunctional natural carriers with broad applications across neurological, gastrointestinal, musculoskeletal, cardiovascular, cutaneous, and regenerative medicine contexts.

Design and modification strategies for targeted delivery

To enhance therapeutic performance, MEVs can be bioengineered through genetic, chemical, or physical modifications that improve targeting specificity, drug loading, and circulation time. Surface modification via conjugation of ligands, peptides, antibodies, or aptamers enables selective delivery to diseased tissues such as tumors, inflamed sites, or injured organs [362]. Similarly, the incorporation of targeting moieties facilitates receptor-mediated uptake, ensuring site-specific release while minimizing systemic toxicity. These approaches have enabled the creation of modified MEVs tailored for neurological, oncological, hepatic, and inflammatory disorders.

For instance, engineered MEVs expressing integrin-binding motifs or receptor-specific ligands can traverse BBB and deliver therapeutic molecules to neurons and glial cells, offering new possibilities in neurodegenerative disease management. The combination of EVs with biomaterials such as hydrogels or 3D-printed scaffolds further allows for localized, sustained drug release and enhanced tissue regeneration particularly relevant to wound healing and bone repair applications [363].

Modified MEVs for drug delivery.

Protein delivery

Modified MEVs have been utilized to deliver protein-based therapeutics, including monoclonal antibodies and receptor decoys, thereby expanding their functional repertoire. Engineered EVS displaying antibody fragments have been shown to bridge cancer cells and immune cells, promoting host anti-tumor immunity. For instance, antibody-modified EVs capable of dual-binding to CD63 on T cells and EGFR on triple-negative breast cancer cells enhanced immune-mediated tumor clearance [364]. Similarly, EVs engineered to express angiotensin-converting enzyme II (ACE2) receptors have functioned as viral decoys, effectively neutralizing SARS-CoV-2 and preventing host cell infection [365]. Other designs, such as transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI)-EVs, demonstrated therapeutic efficacy in lupus models by neutralizing B-cell activators (BLyS and APRIL), reducing serum autoantibodies, and alleviating renal damage [366].

Nucleic acid delivery

MEVs represent a promising platform for delivering therapeutic nucleic acids, including messenger RNA (mRNA), microRNA (miRNA), small-interfering RNA (siRNA), and CRISPR-Cas systems [367]. Owing to their natural biocompatibility and membrane-protective properties, EVs provide a stable and efficient platform for nucleic acid encapsulation, protecting these labile molecules from enzymatic degradation and enhancing their cellular uptake and bioavailability.

Therapeutic nucleic acid agents can be precisely designed based on complementary sequences, enabling selective targeting of genetic and viral pathologies. Unlike protein-based drugs, nucleic acid therapeutics offer rapid adaptability to emerging mutations, such as those seen in recurrent cancers or viral infections [361]. EV-based nucleic acid delivery systems have demonstrated remarkable promise in preclinical studies. For instance, exosome-associated adeno-associated virus (Exo-AAV) vectors, produced from transfected HEK-293T cells, have successfully delivered therapeutic genes to cochlear and vestibular hair cells, partially restoring hearing in genetically deaf mice [368]. Similar delivery to retinal tissues via Exo-AAV achieved efficient intravitreal gene transfer, penetrating both the inner nuclear and outer plexiform layers, thereby supporting retinal repair [369]. In viral infection models, EVs loaded with clustered regularly interspaced short palindromic repeats (CRISPR) Cas9 systems carrying HPV or HBV specific guide RNAs have effectively targeted and cleaved viral DNA within infected host cells, reducing viral persistence and offering a foundation for gene-editing-based antiviral therapy [370].

Collectively, these studies highlight the unique potential of modified EVs including MEVs as versatile carriers for therapeutic nucleic acids. By combining stability, targeting capability, and customizable molecular cargo, EV-based gene delivery systems represent a promising direction for precision medicine and the treatment of previously intractable genetic and infectious diseases.

EV based mRNA therapeutics for tissue regeneration

EV based mRNA delivery is emerging as a powerful approach in regenerative medicine, enabling localized and transient protein expression with high biocompatibility. Current evidence indicates that EV mediated mRNA delivery primarily supports osteogenesis, angiogenesis, and collagen replacement, which are central to tissue repair [371]. Chen et al.. indicated that in bone regeneration, EVs derived from M2 macrophages and loaded with IL-10 mRNA promote osteogenesis by

activating IL-10/IL-10R signaling, enhancing osteoblast differentiation while inhibiting osteoclast activity and bone resorption [372]. In parallel, EV mediated delivery of mitochondrial transcription factor A (TFAM) mRNA enhances mitochondrial oxidative phosphorylation in dental pulp stem cells, further supporting osteogenic differentiation [373]. EV based mRNA delivery also plays a key role in cutaneous regeneration by promoting angiogenesis. Mesenchymal stromal cell derived EVs carrying angiogenic mRNAs activate PI3K/AKT and eNOS pathways, facilitating neovascularization and wound healing [374]. Saliva derived EVs enriched with UBE2O mRNA suppress SMAD6 signaling, activate BMP-2, and further enhance angiogenesis and tissue repair [375]. Additionally, EVs encapsulating collagen mRNA enable sustained dermal collagen expression, restoring collagen levels and reducing photoaging associated wrinkles with minimal inflammation compared to lipid nanoparticle based systems [376]. Advanced tissue engineering strategies have further demonstrated that EVs co-delivering VEGF-A and BMP-2 mRNAs within biodegradable scaffolds synergistically enhance angiogenesis and bone regeneration while limiting off-target distribution [377]. EV based mRNA therapeutics offer a versatile and biocompatible platform for tissue regeneration, supporting coordinated repair of bone, vascular, and extracellular matrix components.

EV based mRNA therapeutics for neurodegenerative diseases

EV mediated mRNA delivery represents a promising therapeutic strategy for neurodegenerative diseases by enabling efficient and targeted gene modulation with minimal immunogenicity. Owing to their endogenous origin, EVs can traverse the BBB, protect labile mRNA cargo, and bypass systemic metabolism, making them particularly suitable for CNS applications. In addition to therapeutic delivery, EV associated mRNAs also hold potential as biomarkers for early diagnosis and disease monitoring. EV based mRNA approaches are being actively explored in disorders such as Parkinson's disease (PD) and Alzheimer's diseases (AD). Age-dependent differences in EV mRNA profiles have revealed increased levels of pro-inflammatory mRNAs (including IL-6, TNF-α, and IL-12) in EVs derived from stimulated immune cells in older individuals, providing mechanistic insights into AD associated neuroinflammation. Advances in EV engineering have further strengthened their therapeutic relevance. Engineered EVs with enhanced biogenesis, brain targeting ligands, and improved cytosolic delivery have been successfully used to deliver catalase mRNA in PD models, resulting in reduced neuroinflammation and functional therapeutic outcomes. EV based mRNA therapeutics play a significant role in CNS communication and offer a versatile platform for treating neurodegenerative diseases, although challenges related to delivery efficiency, safety, and long-term efficacy must still be addressed [371].

EV-based mRNA therapeutics for infectious diseases

EVs have emerged as an efficient and biocompatible platform for mRNA delivery in infectious diseases, overcoming key limitations of conventional antiviral therapies and synthetic carriers. Their endogenous origin confers low immunogenicity, enhanced stability of encapsulated mRNA, and intrinsic targeting capabilities, supporting safe and sustained gene expression. In HIV 1 infection, EV based mRNA strategies have been explored to suppress viral transcription using epigenetic "block and lock" approaches. EVs delivering mRNAs encoding engineered zinc finger based methyltransferases targeting the HIV 1 long terminal repeat region have shown potential to repress viral gene expression and limit disease progression, offering a promising alternative to lifelong antiretroviral therapy [378].

EV based mRNA vaccines have gained particular attention during the COVID-19 pandemic. Compared with lipid nanoparticles, EVs exhibit minimal cytotoxicity, fewer adverse effects, and prolonged protein expression. Preclinical studies demonstrate that EVs carrying mRNAs encoding SARS-CoV-2 antigens elicit robust humoral and cellular immune responses, including strong $CD4^+$ and $CD8^+$ T-cell activation, with favorable safety profiles. EV based mRNA therapeutics represent a versatile and scalable platform for infectious disease treatment and vaccination, enabling targeted delivery, alternative administration routes, and enhanced immune activation with improved safety [371].

Chemotherapeutic drug delivery

Modified MEVs have also demonstrated efficacy in the targeted delivery of chemotherapeutic agents such as paclitaxel, doxorubicin, and sorafenib, enabling enhanced drug stability, bioavailability, and tumor-specific targeting. By leveraging their intrinsic biocompatibility and capacity for functional modification, EVs can encapsulate hydrophobic or labile compounds, improving pharmacokinetics while reducing systemic toxicity [361].

Several studies have demonstrated the efficacy of EV-mediated chemotherapeutic delivery. Haney et al. successfully loaded paclitaxel (PTX) and doxorubicin (DOX) into macrophage-derived EVs, achieving potent anticancer effects in a mouse model of pulmonary metastases [379]. Similarly, Zhang et al. employed red blood cell-derived EVs (RBC-EVs) co-loaded with DOX and sorafenib (SRF), which produced superior therapeutic outcomes in hepatocellular carcinoma models compared to conventional drug administration [380]. These findings underscore the potential of EV-based systems to

enhance intratumoral drug accumulation and therapeutic selectivity.

The versatility of EVs extends to poorly soluble and unstable drugs such as curcumin. Loading curcumin into EVs produced a formulation (cGRDExo-cur) with significantly improved anti-inflammatory efficacy and bioavailability in post ischemic mouse models [381]. Furthermore, placental mesenchymal stem cell (MSC) derived EVs loaded with DOX and functionalized with carboxylated Fe_3O_4 nanoparticles exhibited magnetic targeting capability, allowing precise accumulation within breast tumors under an external magnetic field [382]. This approach markedly increased local drug concentration while minimizing systemic exposure and off-target effects.

Collectively, these findings demonstrate that functionalized and modified EVs including MEVs can serve as efficient, biocompatible nanocarriers for chemotherapeutic agents. Through rational engineering and targeted delivery strategies, they hold significant promise for improving the efficacy and safety of cancer therapies by enabling precise, lesion-specific treatment with minimized adverse effects.

Targeted treatment with modified EVs

Leveraging their capacity for precise drug loading and targeted delivery, modified EVs can traverse biological barriers and deliver therapeutic cargo to specific tissues, highlighting their potential for treating complex disorders, including brain and liver disorders and tumor therapy.

EVs and the brain

Brain disorders remain a major clinical challenge due to the restrictive nature of the BBB. Modified EVs, with their inherent biocompatibility and ability to cross the BBB, offer a promising strategy for targeted brain therapy. EVs facilitate communication between neurons, glia, and vascular cells, regulate synaptic activity, maintain neurovascular integrity, and influence neurodegenerative processes [361]. Their neuroprotective and regenerative potential, demonstrated across multiple studies, underscores their promise as nanoscale therapeutic agents for brain disorders.

EVs and the blood brain barrier

The BBB represents one of the most selective and tightly regulated biological interfaces, maintaining CNS homeostasis while restricting the entry of most therapeutic molecules [383]. It is primarily composed of brain microvascular endothelial cells (BMECs), pericytes, astrocytes, and tight junction proteins such as occludin and claudin-5, which together limit paracellular diffusion and regulate transcellular transport (419).

EVs have demonstrated the remarkable capability to traverse the BBB, offering a promising route for CNS drug delivery. Integrins present on EV membranes (e.g., αvβ3, α4β1) recognize vascular cell adhesion molecule-1 (VCAM-1) and fibronectin (FN) on BMECs, initiating adhesion and subsequent endocytosis [385]. Upon internalization, EVs induce caveolae-mediated uptake through caveolin-1 and dynamin GTPase, forming caveosomes that undergo exocytosis into the cerebral parenchyma [386]. Once across the BBB, EVs interact with neurons, astrocytes, and microglia through ligand receptor recognition, releasing their bioactive cargos (proteins, miRNAs, and lipids) via membrane fusion or endosomal pathways to modulate neuronal function and neuroinflammation [387].

Experimental evidence supports the bidirectional transport of EVs across the BBB, highlighting their physiological and therapeutic relevance. For instance, erythrocyte derived EVs have been shown to transfer α-synuclein through transcytosis [388], while endothelial cell-derived EVs can cross from the periphery into the CNS, carrying signaling molecules and serving as potential biomarkers of BBB integrity [389]. Furthermore, brain endothelial cell-specific EVs isolated under different pathophysiological conditions have revealed new receptor targets and diagnostic signatures, suggesting their dual role in biomarker discovery and therapeutic delivery.

EVs and brain disorders

EVs are increasingly recognized as key mediators in the pathophysiology and treatment of neurological disorders such as stroke, Alzheimer's disease (AD), traumatic brain injury (TBI), and schizophrenia [390].

In stroke, EVs exert neuroprotective effects primarily through anti-inflammatory and pro-regenerative mechanisms rather than direct tissue repair. By downregulating inflammatory cascades, EVs reduce BBB permeability and the extent of secondary neuronal injury. Astrocyte-derived EVs enriched with prostaglandin D_2 synthase promote axonal growth and facilitate post stroke recovery [391].

In AD, the therapeutic and diagnostic potential of EVs is particularly well documented. EVs can capture β-amyloid (Aβ) oligomers via surface proteins, neutralizing their synaptotoxic effects. Mesenchymal stem cell-derived EVs (MSC-EVs) containing Aβ-degrading enzymes further enhance Aβ clearance and mitigate plaque accumulation [387]. In addition, EVs from curcumin-pretreated cells (Exo-cur) inhibit Tau protein hyperphosphorylation by activating the AKT/GSK-3β signalling axis, thus preventing neuronal apoptosis and alleviating cognitive decline [383]. EVs also attenuate neuroinflammation by reducing nitric oxide release, suppressing TNF-α and IL-1β expression, and upregulating anti-inflammatory cytokines such as IL-10 [392]. Beyond

therapy, astrocyte-derived EVs (ADEs) have shown diagnostic potential in differentiating AD and frontotemporal dementia through quantitative proteomic profiling.

In traumatic brain injury, EVs serve both diagnostic and therapeutic roles. Their cargo reflects the cellular origin and state of injury, making them valuable biomarkers for disease staging. Intranasal delivery of MSC-EVs in TBI animal models has been shown to promote neurofunctional recovery, suppress NLRP3 inflammasome activation, inhibit p38 MAPK signalling, and prevent microglial polarization toward the pro-inflammatory M1 phenotype, collectively improving behavioural and cognitive outcomes [393].

In schizophrenia, the intranasal administration of MSC-derived EVs in phencyclidine (PCP)-induced rodent models has demonstrated behavioural and neurochemical improvements, including restored social interaction, normalization of repulse inhibition, and preservation of parvalbumin-positive GABAergic interneurons in the prefrontal cortex. Moreover, MSC-EVs reduce glutamate levels in cerebrospinal fluid, mitigating excitotoxicity and neuronal dysfunction [394].

Collectively, these findings substantiate the potential of EVs particularly modified and surface-engineered formulations to navigate complex physiological barriers such as the BBB and exert disease-modifying effects in diverse neurological disorders. The successful demonstration of targeted CNS delivery underscores the broader applicability of EV-based strategies for other pathologies requiring precise site-specific drug localization, such as tumors. The following section elaborates on the targeted engineering and mechanisms of modified EVs in tumor therapy.

Modified EVs for tumor therapy

Building on the demonstrated ability of EVs to traverse complex physiological barriers such as the BBB and deliver bioactive cargo with high specificity, engineered EVs have emerged as a promising platform for the targeted treatment of tumors. Modified EVs can inhibit tumor growth and metastasis through multiple mechanisms, including the delivery of pro-apoptotic molecules, modulation of the tumor microenvironment, and activation of antitumor immune responses. Studies in models of breast and lung cancer have shown that engineered EVs can induce apoptosis in tumor cells, disrupt immune tolerance, and enhance the efficacy of personalized cancer therapy.

Tumor progression and metastasis are driven by intricate intercellular communication. The tumor microenvironment (TME) sends signals locally and to distant pre-metastatic niches (PMNs), promoting metastatic colonization and rapid tumor growth. EVs, as natural carriers of bioactive molecules, play a dual role: they can facilitate tumor progression by promoting proliferation, angiogenesis, immune evasion, and metabolic regulation, when engineered, they serve as highly effective therapeutic vehicles. Tumor-secreted EVs deliver miRNAs and other molecules to stromal cells, while EVs derived from bone marrow MSCs carrying miR-23b can induce dormancy of breast cancer cells in the PMN, highlighting the modulatory potential of EV cargo [361].

Therapeutic engineering of EVs further enhances antitumor effects. For example, MSC-derived EVs modified to express TNF-related apoptosis-inducing ligand (TRAIL) on their surface MSCT-EVs selectively trigger apoptosis in cancer cells in a dose-dependent manner [395]. Similarly, loading cytotoxic agents like paclitaxel into autologous prostate cancer cell-derived EVs significantly improves their anticancer efficacy [396]. Tumor-derived signalling molecules, including cytokines, immunological checkpoint stimulators, and microvesicles, often promote immune tolerance, enabling cancer cells to escape immune surveillance. Overcoming this immune evasion is significant for effective cancer immunotherapy. Functional dendritic cells (DCs) can elicit robust antitumor T-cell responses; however, their activity is sensitive to the immunosuppressive tumor microenvironment [397]. EVs derived from genetically modified or antigen-loaded DCs commonly termed Dexosomes (Dex) retain immunogenic activity under changing conditions and have demonstrated therapeutic potential in preclinical studies as well as Phase I/II clinical trials for advanced malignancies, including metastatic melanoma and inoperable non-small cell lung cancer.

Overall, the targeted delivery, immune modulation, and tumor-inhibitory capabilities of modified EVs have been firmly established. This platform not only addresses the challenges of tumor heterogeneity and immune evasion but also provides a foundation for precision therapy in other organs.

Engineered EVs for vaccination in cancer and infectious diseases

Engineered EVs are increasingly recognized as a promising and versatile vaccine delivery platform due to their natural biocompatibility, nanoscale size, intrinsic immunological functions, and capacity for precise molecular engineering. Advances in EV bioengineering have enabled efficient loading of antigens either as surface displayed proteins or as encapsulated antigen-encoding mRNAs, using both endogenous strategies (genetic modification of parental cells) and exogenous post isolation approaches. Genetic engineering methods commonly exploit EV-associated scaffolds such as tetraspanins, Lamp2b, syntenin, or viral fusogenic proteins to ensure stable antigen incorporation while preserving native antigen conformation, a significant determinant of effective immune recognition [398]. In parallel, post

formation engineering techniques, including bioconjugation, lipid insertion, and physical permeabilization, provide greater control over antigen density and loading efficiency, thereby expanding the flexibility of EV based vaccine design [399]. Preclinical studies have demonstrated that engineered EV vaccines can elicit robust and durable immune responses across both cancer and infectious disease models. In cancer immunotherapy, dendritic cell derived EVs and tumor derived EVs retain key antigen-presenting and co-stimulatory molecules, enabling efficient priming of antigen-specific $CD8^+$ and $CD4^+$ T-cell responses [400]. These EV vaccines have shown enhanced antitumor efficacy, particularly when combined with immune adjuvants or immune checkpoint inhibitors, highlighting their ability to modulate the tumor immune microenvironment. Similarly, in the context of infectious diseases, EV-based vaccines engineered to deliver viral antigens or mRNA cargos have demonstrated strong humoral and cellular immunity against pathogens such as influenza virus, HIV, and SARS-CoV-2, with additional advantages including improved mucosal immunity, reduced reactogenicity, and enhanced stability relative to conventional lipid nanoparticle–based systems [401]. Collectively, these findings underscore the potential of engineered EVs as a modular and broadly applicable vaccine platform capable of overcoming several limitations of current vaccine delivery technologies. The ability to tailor antigen presentation, co-deliver immunostimulatory components, and achieve tissue-specific biodistribution positions EV-based vaccines as a compelling strategy not only for cancer and infectious diseases but also for expanding EV applications to other therapeutic areas.

The following Table 5 presents detailed information regarding the drug delivery applications of MEVs.

Translational insights from clinical studies on MEVs

MEVs are emerging as a promising class of bioactive nanocarriers due to their natural origin, biocompatibility, and inherent ability to deliver proteins, lipids, and nucleic acids to target tissues. Preclinical studies have highlighted MEVs' immunomodulatory, anti-inflammatory, and regenerative properties, paving the way for translational applications in human diseases. Clinical studies are beginning to explore their safety, tolerability, and therapeutic potential in diverse conditions, including inflammatory bowel disease, neonatal jaundice, metabolic disorders, and infant health modulation. Specifically, whey protein MEVs (WPMDEs) have been evaluated in Phase 1 trials for safety and tolerability in both healthy adults and patients with ulcerative colitis (UC). The studies indicate potential anti-inflammatory effects, improvement in patient-reported outcomes, and modulation of disease biomarkers such as faecal calprotectin. Similarly, breast MEVs have been investigated for their role in neonatal health, including jaundice and postnatal metabolic regulation. Collectively, these case studies provide early clinical and translational evidence supporting the potential of MEVs as safe, bioactive adjunct therapies, warranting further controlled trials to establish efficacy. Table 6 highlights the clinical and translational evidence of MEVs, summarizing key preclinical and clinical studies that demonstrate their therapeutic potential, safety, and applicability across diverse biomedical domains.

Challenges and future perspectives

In recent years, MEVs have emerged as promising therapeutic candidates due to their inherent biocompatibility, stability, and ability to transport diverse bioactive molecules, including proteins, lipids, and microRNAs. These bioactive cargos confer anti-inflammatory, immunomodulatory, and regenerative effects, making MEVs attractive for therapeutic, nutraceutical, and drug delivery applications. Despite their potential, the effective clinical translation of MEVs is constrained by several interconnected challenges. Biological variability arising from milk source, lactation stage, and animal health can influence vesicle composition, heterogeneity, and receptor-mediated uptake, affecting reproducibility and functional outcomes. Pharmacokinetics and biodistribution are difficult to predict due to rapid clearance, non-specific tissue absorption, and degradation in biological environments. Vesicle stability is further threatened by pH fluctuations, enzymatic breakdown, and storage conditions, potentially compromising structural integrity and bioactivity. Technical limitations also persist in scalable isolation, purification, and characterization methods. Current approaches often yield low quantities, co-isolate contaminants, and compromise vesicle integrity. Drug loading efficiency is limited, with challenges in achieving controlled release and preventing premature cargo leakage. Surface modification and functionalization are further complicated by variability in vesicle markers, affecting targeting and reproducibility. Moreover, regulatory frameworks for MEVs, whether classified as nutraceuticals or therapeutic biologics, remain undefined, posing additional translational barriers. Preliminary clinical studies, including WPMDE1 and WPMDE2, have demonstrated favorable safety profiles and potential efficacy, supporting the translational relevance of MEVs. Opportunities exist to expand their applications to inflammatory, metabolic, neurodegenerative, and neonatal disorders. Advances in bioengineering allow for optimized cargo loading, targeted delivery, and integration with biomaterials such as hydrogels or 3D-printed scaffolds. Omics-based approaches may further enable personalized MEV therapies, enhancing efficacy and safety. However, potential threats include regulatory

Table 5 MEV case studies: Applications, conditions and insights

Drug	Pathological condition	Characterization of EV	Discussion	Reference
		Protein Markers/mRNA		
Doxorubicin	Triple negative breast cancer (TNBC)	TSG101, tetraspanins CD63, CD82, CD9	BMEVs enhance the sensitivity of TNBC cells to doxorubicin by reducing metabolic activity and cell viability, suppressing interferon-responsive gene products and STAT signaling pathways, thereby improving the efficacy of chemotherapeutic treatment in preclinical tumor models	[402]
TNF-α siRNA delivery	Inflammatory bowel disease (IBD)	TSG101 CD9	MEV encapsulated TNF-α siRNA enables stable oral delivery to inflamed intestinal tissues, suppresses TNF-α expression, and alleviates colitis symptoms in DSS-induced IBD models, highlighting MEVs as an effective oral gene-therapy platform for IBD	[71]
Glycyrrhetinic acid (GA)	Idiopathic pulmonary fibrosis (IPF)	CD 63 CD 9	Inhalable MEVs loaded with glycyrrhetinic acid attenuate pulmonary fibrosis by suppressing TGF-β1/Smad3 signaling and pro-inflammatory cytokines, significantly reducing fibrosis and improving lung function in bleomycin-induced IPF models at a reduced drug dose	[403]
RNA	-	CD63, CD81, ICAM, TSG101, ALIX	MEV - liposome hybrid nanovesicles (hybridosomes) enable efficient and stable oral siRNA delivery, exhibiting enhanced intestinal stability, reduced cytotoxicity, improved epithelial permeation, and superior siRNA loading compared with conventional cationic liposomes	[405]
Curcumin	Chronic liver disease		Saponin-assisted loading of curcumin into MEVs markedly enhances drug encapsulation and selectively increases cytotoxicity toward tumor cells, while significantly reducing liver injury and fibrosis markers in experimental liver fibrosis models	[405]
Insulin	Diabetes	CD63, CD81 and TSG101	Insulin loaded MEVs enable effective oral peptide delivery by enhancing gastrointestinal stability, mucus penetration, and multi-pathway intestinal uptake, producing a sustained hypoglycemic effect in type I diabetic rat models	[406]
α-Mangosteen	Bacterial infections	Flotillin-1 and ESCRT protein TSG-101	Phosphatidylserine functionalized MEVs enhance the solubility and oral delivery of poorly soluble BCS class II/IV drugs, promote phagocytic targeting, and significantly improve antibacterial efficacy in cellular and animal infection models	[407]
Paclitaxel	Breast cancer		Folic acid functionalized BMEVs enable targeted oral delivery of paclitaxel, enhancing cellular uptake, sustained drug release, and cytotoxic efficacy, with significantly reduced IC_{50} and improved inhibition of cancer cell migration	[408]
Paclitaxel	Lung tumour	-	Orally administered paclitaxel-loaded MEVs demonstrate high gastrointestinal stability, significantly greater tumor growth inhibition than conventional paclitaxel, and reduced systemic and immunogenic toxicity, supporting MEVs as a viable alternative to intravenous chemotherapy delivery	[163]
Doxorubicin anthracene endoperoxide	Oral squamous cell carcinomas	CD9, CD63, TSG101	A pH and light responsive MEV based nanoplatform enables controlled doxorubicin release and enhanced photodynamic therapy via ROS generation, demonstrating effective and biocompatible treatment of oral squamous cell carcinoma in vitro and *in vivo*	[409]
-	Bone generation	IL-10 mRNA	This study demonstrates that M2 derived EVs exert osteoprotective effects by delivering IL-10 mRNA to bone marrow derived cells, activating the IL-10/IL-10R pathway to promote osteogenesis and suppress osteoclastogenesis, thereby highlighting a novel therapeutic strategy for periodontitis	[372]

uncertainty, competition from synthetic nanocarriers with established manufacturing pipelines, variability in clinical outcomes, and scalability constraints.

A strategic roadmap for MEV research emphasizes sequential steps to bridge experimental research and clinical application. Standardization of isolation, purification, and characterization protocols including assessment of size, zeta potential, and molecular cargo is essential for reproducibility. Preclinical studies should systematically investigate cellular uptake, biodistribution, pharmacokinetics, toxicity, and functional efficacy in disease-relevant models to provide mechanistic insights. Early-phase clinical trials in healthy volunteers and patient populations are significant to establish safety, tolerability, and efficacy, complemented by biomarker analyses for translational relevance. Therapeutic optimization through cargo engineering and combination with advanced biomaterials can enhance targeted delivery and sustained release. Engagement with regulatory authorities, alongside development of scalable, GMP-compliant manufacturing processes, is pivotal for successful translation. Finally, long-term studies assessing safety, immunogenicity, and clinical outcomes will support broader applications of MEV-based interventions, ultimately enabling their progression from experimental studies to clinically viable therapeutics.

Table 6 Clinical and translational evidence of MEVs

Reference/Clinical Trials ID	Study/Trial	Population	EV Source & Product	Study Type/Phase	Intervention & Dose	Primary Outcomes/Endpoints	Secondary outcomes	Key findings/Translational relevance
NCT06742203(451)	WPMDE1	Healthy adults ($n=13$)	Whey protein milk-derived exosomes	Phase 1, open-label, single-center	Oral, ascending doses over 7–30 days	Tolerability, usability	Daily symptom tracking, vital signs, blood sampling	Demonstrated good tolerability and safety; established dose range for future trials targeting IBS and IBD
NCT06755021(452)	WPMDE2	UC patients ($n=100$; mild–severe)	Whey protein milk-derived exosomes (powder; 5 g & 10 g)	Phase 1B, open-label, multicenter	Oral, twice daily for 8 weeks, add on to standard therapy	Safety, tolerability, changes in disease severity	QoL (IBDQ-32), fecal calprotectin, endoscopic assessment (optional), stool frequency, abdominal pain	Preliminary evidence for anti-inflammatory and immunomodulatory effects; potential adjunct therapy for UC; supports further controlled trials
NCT06502847(453)	Neonatal Jaundice	Infants with ABO incompatibility ($n=45$)	Breastmilk & serum exosomes	Observational	Exosome profiling	Correlation of exosome profiles with jaundice severity	Laboratory markers, clinical outcomes	Suggests diagnostic and predictive value of exosomes in neonatal hyperbilirubinemia
NCT06892483(454)	Breastmilk Response to Exercise	Postpartum mothers and infants ($n=60$)	Breastmilk exosomes	Observational	Pre-pregnancy BMI & maternal exercise	Infant growth & metabolic markers	Breastmilk composition changes, immune factors	Provides mechanistic insight into maternal lifestyle effects on milk exosome content and infant health
NCT04527536(455)	BMJ Diagnostic Study	Infants with breast milk jaundice ($n=300$)	Breastmilk & fecal exosomes	Observational, nested case-control	Diagnostic test	ROC analysis of fecal miRNA and intestinal flora	Early-onset vs. late-onset BMJ comparison	Demonstrates feasibility of exosome-based biomarkers for early diagnosis
NCT04924504(456)	SIR-MET	Pregnant women with T2DM or GDM ($n=24$)	Maternal blood & breastmilk exosomes	Observational	None (profiling study)	Insulin sensitivity, hormonal & inflammatory markers	Placenta, maternal and umbilical cord blood, breastmilk metabolomics	Correlation of exosome content with maternal/fetal insulin resistance and metabolic outcomes; supports mechanistic understanding for translational applications

Conclusion

MEVs are biologically significant nano-sized extracellular vesicles that facilitate intercellular communication and bioactive molecule transfer. Their unique biogenesis, stability, and uptake mechanisms make them promising candidates for nutrient transport, drug delivery, and therapeutic applications. Compared with EVs derived from cultured mammalian cells, stem cells, or immune cells, MEVs offer unique advantages in terms of safety, scalability, robustness, and translational feasibility. A major strength of MEVs is their excellent biocompatibility and safety profile. As natural components of the human diet, MEVs exhibit minimal immunogenicity, low systemic toxicity, and good tolerability, including in cross-species administration. In contrast, cell derived EVs may carry risks associated with donor cell variability, unwanted bioactive cargo, and batch to batch inconsistency. MEVs also demonstrate superior physicochemical stability, maintaining vesicle integrity under acidic pH, enzymatic digestion, thermal stress, and varied storage conditions, whereas many cell-derived EVs require ultra-cold storage and are more vulnerable to degradation during processing. MEVs further outperform other EV sources in terms of scalability and cost effectiveness. Milk, particularly bovine milk contains exceptionally high concentrations of EVs, enabling gram-scale yields from liter scale volumes using established dairy infrastructure. This production advantage contrasts with the limited yield and high cost associated with EVs derived from cell culture systems that rely on bioreactors and complex manufacturing workflows. Consequently, MEVs represent a more practical platform for large scale pharmaceutical and nutraceutical applications. Functionally, MEVs serve as versatile nanocarriers capable of incorporating diverse therapeutic payloads, including small molecules, proteins, and nucleic acids. Accumulating evidence indicates that MEVs enhance the stability, bioavailability, and systemic exposure of encapsulated cargo, particularly following oral administration, outperforming free drugs and several synthetic nanocarriers. In addition, MEVs possess intrinsic bioactivity due to their conserved cargo of miRNAs, proteins, and lipids, which regulate immune responses, inflammation, epithelial barrier integrity, and cell survival. This endogenous biological activity can synergize with loaded therapeutics, offering functional benefits beyond passive drug delivery.

However, their clinical and research utility is hindered by challenges in isolation, characterization, stability, and surface modification. Milk contains a complex mixture of proteins, lipoproteins, and casein micelles, making the MEVs isolation process intricate. The choice of an isolation method significantly impacts yield, purity, and functionality. A combination of SEC, density gradient ultracentrifugation, and immunoaffinity capture could improve MEVs yield and purity while maintaining their biological activity. Advancements in high-throughput isolation methods, standardized characterization techniques, and innovative surface engineering approaches are essential to optimize their potential. Combining density gradient ultracentrifugation (UC) or SEC with advanced profiling tools like nano-flow cytometry, Cryo-EM, and RNA-seq can improve purity, reproducibility, and functional assessment. Stability and pharmacokinetics considerations are influenced by physicochemical, environmental, and biological factors, including pH fluctuations, enzyme breakdown, and storage conditions. Encapsulation strategies such as PEGylation, lyophilization with cryoprotectants, and biomimetic coatings can help preserve stability and bioactivity under physiological conditions. Accurate characterization is essential for understanding the size, morphology, cargo composition, and surface markers of MEVs. Standard techniques face various limitations, but advanced techniques such as single-vesicle analysis, label-free biosensing, and AI-driven nanoparticle profiling can improve the accuracy and reproducibility of MEVs characterization. Surface engineering for targeted delivery is key to enhancing MEVs stability, circulation time, and tissue-specific delivery. Different modification strategies come with trade-offs, such as PEGylation, Ligand Conjugation (Aptamers, Antibodies, Peptides), Genetic Engineering of Parent Cells, and Biomimetic Coatings (Hybrid Vesicles, Lipid Insertion). By addressing these challenges through cutting-edge bioengineering and analytical innovations, MEVs can be fully leveraged as next-generation nanocarriers for drug delivery, immune modulation, and diagnostics, driving their translation into clinical and industrial applications. Innovations in machine learning-based MEVs profiling, AI-assisted drug loading optimization, microfluidic-based isolation, and bioengineered MEVs-producing cells will be pivotal in overcoming current limitations. Standardizing protocols and obtaining regulatory approvals will further enable the transition of MEVs-based nanomedicine from research to real-world applications. By addressing these key challenges, MEVs can be fully harnessed as next-generation nanocarriers, revolutionizing personalized medicine, targeted therapy, and industrial biotechnologies. In conclusion, the development of MEVs necessitates addressing several challenges including large-scale purification, stable milk source procurement, raw milk quality control systems, standardized production processes, and stringent quality standards. In the coming years, both natural functional and engineered MEV products will undergo clinical development and transformation.

Author contributions

Mahananda R. Prabhu: Writing – review and editing, writing – original draft, Visualization, Methodology, Investigation, Data curation. Dinesh Upadhya:

Reviewing, editing, Validation, Conceptualization. Sri Renukadevi Balusamy: Critical editing, Supervision, Review & Editing. Shadi Rahimi: Funding and Overall critical assessment, Harishkumar Madhyastha: Review & editing, Conceptualization. Anup Naha: Review & editing, Validation, Supervision, Formal analysis. Haribalan Perumalsamy: Manuscript review & Editing. Sneha Sunderraj: Formal analysis. Akhilesh Dubey: Review and editing. Srinivas Hebbar: Review & editing, Visualization, Methodology, Investigation, Data curation, Validation, Conceptualization, Formal analysis.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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