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Harnessing the full potential of extracellular vesicles as drug carriers

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Abstract

Extracellular vesicles are natural delivery systems widely implicated in cellular communication. However, to fully utilize these vehicles as nanocarriers, we must explore various methods to modify their applicability as drug delivery vehicles. In this review, we outline and discuss techniques to engineer extracellular vehicles for enhanced loading, targeting, circulation, and tracking. We highlight cutting-edge methods to amplify extracellular vesicle secretion and production and optimize storage conditions to improve their clinical suitability. Moreover, we focus on reverse engineering as an important step in controlling their biological function. By taking a reductionist approach to characterize and understand the individual components of these carriers, we can not only elucidate complex mechanisms of action but also advance the field through the creation of synthetic drug delivery vehicles. Finally, we propose current challenges and future directions of the field.

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Introduction

There is an ever-increasing need for biologically compatible drug delivery systems that can transport and protect therapeutic cargo throughout the body. Over the last decade, extracellular vesicles (EVs) have garnered significant attention as drug carriers and cell-replacement therapies. Extracellular vesicles are nonreplicating, lipid-bilayer particles naturally released by almost all cells in the human body [1], as well as in many plants [2] and animals [3]. In this review, we cover small EVs, also known as exosomes that are 50−150 nm in size. While it was previously thought that they were simply cellular waste carriers, it is now clear they are extensively implicated in cell-to-cell communication, even between distant locations [4,5]. In addition, EVs can cross biological barriers and are filled with a variety of nucleic acids, lipids, and proteins that can exert biological function, making them natural materials for cell-free therapeutics. Various studies have identified EVs as mediators for regenerative wound healing, priming vehicles for immune cell infiltration at tumor sites, as easily collectible biomarkers for many indications, and much more [6,7]. Currently, there are three active clinical trials and several trials in the enrolling and recruiting phases.

While many types of EVs can be used to promote healthy cellular function, other types, such as tumor-derived vesicles, have also been identified as key players in various pathogenic pathways. The mechanisms behind the EV function and these divergent roles are still poorly understood [8]. Additional challenges for their potential clinical use include their poor loading efficiencies, their rapid clearance from circulation, and their scalability [9]. To address these barriers, EVs must be engineered to achieve optimal effects. By characterizing EV populations from various donor cells and identifying their most potent factors, EVs can be manipulated and fine-tuned to optimize their therapeutically beneficial components. Additionally, reverse engineering of EVs can be utilized to design synthetic biomimetic systems, widening the clinical applicability of these systems.

In this review, we provide an overview of the current methods in which EVs have been modified to overcome their current barriers as drug delivery vehicles. We further discuss current challenges in the field and cutting-edge strategies that can be exploited in the future for therapeutic EV drug delivery.

Cargo loading

Although EVs are endogenously loaded with a wide set of biologically active molecules, it is often desired to modulate EV function through loading with therapeutic drug molecules. However, low loading efficiency remains a challenge for therapeutic use. The efficiency of vesicle loading is often dependent on the physicochemical characteristics of the type of cargo used. Below, we summarize the biochemical properties of each major cargo type and techniques used to optimize their loading.

Loading of nucleic acids

Nucleic acid loading is primarily dictated by their negative charge, large size, and stability [10]. The negative charge of nucleic acids and the negative charge of the EV membrane leads to electrostatic repulsion and poses major challenges for effectively loading nucleic acids into EVs. One way to address this challenge is by loading nucleic acids during EV biogenesis. Studies have shown that overexpressing nucleic acids in the parent cells causes them to also enrich into EVs [11]. However, loading efficiency remains extremely low. Therefore, active loading methods have been developed to increase loading efficiency. Wang et al. showed that when mesenchymal stem cells (MSCs) were electroporated with miR-101a, this RNA was detected in EVs secreted from MSCs. When injected intravenously in mice in a myocardial infarction model, these miR-101a loaded EVs robustly improved cardiac function and decreased infarct size [12].

Despite successes, some active loading methods are associated with additional challenges. For example, loading nucleic acids directly into EVs by electroporation has been shown to cause nucleic acids to become insoluble and form precipitates. Kooijmans et al. observed that even in the absence of EVs, significant levels of electroporated siRNA remained as 100 nm aggregates after isolation by ultracentrifugation. Overall, they found the loading efficiency to be extremely low at levels less than 0.05% as opposed to previously determined values of up to 25% [13]. Later studies by Sachdev et al. showed a similar effect with the size-dependent electroporation of DNA cargo into cells. Specifically, they observed that DNA molecules 15 bp in size experienced no aggregation and were able to translocate into the cytoplasm of cells freely. DNA between 25 and 100 bp, however, form rod-like molecules that aggregate easily and are unable to move through the membrane's bilayer [14]. Jeyaram et al. addressed these challenges by protonating the membrane of EVs to create a pH gradient in the membrane. By reducing the internal pH of EVs, they discovered that RNA was loaded approximately four times more efficiently. They also found that room temperature electroporation with 2-h incubation times created optimized loading efficiencies. At warmer and longer time points, decreased loading, likely due to RNA degradation or loss of the pH gradient, was observed [15].

Other methods for loading nucleic acids into EVs utilize sequence-specific cellular sorting machinery. These studies hypothesize that there are common motifs within highly enriched RNAs that bind to proteins to facilitate sorting into EVs before secretion. Studies by Villaroya Beltri et al. identified two short motifs (GGAG and CCCU) that are overrepresented in EV miRNAs, which they termed EXO-motifs. They found that when miRNAs were modified to include these short motifs, the mutated miRNAs showed an increase in EV loading by more than 3-fold. Furthermore, they found that hnRNPA2B1 was highly bound to the EXO-motifs, and hnRNPA2B1 SUMOylation in EVs mediates its capacity to bind and sort miRNAs containing the motif [16]. Temoche-Diaz et al. examined RNA compositions of two distinct EV populations, vesicular High-Density populations (vHDs) and vesicular Low-Density populations (vLDs), secreted from MDA-MB-231 cells and their unique miRNA sorting mechanisms. They discovered that vHDs were highly enriched with miRNAs that were depleted in vLDs but also contained similar levels of the vLD enriched miRNAs. This suggested that the vHDs utilized a selective sorting mechanism, whereas the other population did not. Furthermore, they identified miR-122 as a top candidate for selective sorting into vHD EVs. They found that the protein Lupus La bound tightly to this miRNA through two motifs (UUU and UGGA) and served as a mediator for its EV sorting [17]. As studies continue to identify sequences and proteins implicated in vesicle loading, mechanisms behind EV function can become further elucidated.

Loading of proteins

Proteins are primarily loaded into EVs through pre-EV formation via two methods. Firstly, plasmids encoding for the protein of interest can be transfected or simply loaded into the parent cell, and the protein will be overexpressed. During multivesicular body biogenesis, cytosolic proteins can then be encapsulated within the vesicles and released from the cell within EVs. This loading can be random, inefficient, and is largely limited to proteins with extracellular mechanisms of action [18,19]. In other approaches, recombinant plasmids with the protein of interest fused to a membrane protein are transfected for EV membrane expression. Yim et al. have enhanced the flexibility of membrane-mediated loading of soluble proteins by modifying both the protein cargo and EV membrane proteins, terming this method: exosomes for protein loading using optogenetically reversible protein–protein interaction (EXPLOR). First, they transfected the parent cells with a recombinant plasmid encoding for a fusion protein of CD9 and CIBN, a CRY2 interacting protein. Then they created a fusion protein with the protein cargo and

CRY2, a photoreceptor. By incubating the transfected cells in the presence of blue light, the protein cargo was able to transiently bind to the cell membrane, which transitions to the EV inner membrane during biogenesis. With this novel method, the proteins can detach in the absence of light and be loaded into EVs over 20 times higher than simple overexpression [18]. However, this method's loading efficiency is largely dependent on the amount of DNA used, cell type, and other transfection-specific factors. Alternative approaches may circumvent these factors. For example, when comparing passive incubation at room temperature, permeabilization with saponin, freeze−thaw cycles, sonication, and extrusion, Haney et al. found that sonication and extrusion allowed for the greatest loading efficiency of the enzymatic Parkinson's drug, catalase, 26.1%, and 22.2% (w/w) respectively. Importantly, they found that catalase structure was preserved upon loading, and no enzymatic function was lost using sonication, while free catalase function was reduced to 14% [20]. However, studies by Sukre et al. found that ultrasonicated bovine milk EVs lost expression of traditional markers, CD63 and ALIX, via Western blot detection. They also observed that sonicated EVs had significantly reduced miRNA levels and had reduced uptake by target cells [21]. Therefore, this method may not be recommended for protein cargo loading.

Researchers have also investigated the sequence-specific protein-mediated loading of proteins into EVs. Sterzenbach et al. demonstrated that target proteins could be labeled with a WW tag, which binds the L-domain-containing protein Ndfip1, which has been implicated in the recruitment of the endosomal sorting complex required for transport (ESCRT) components and multivesicular body (MVB) formation [22]. They hypothesized that WW-fusion proteins would be able to bind Ndfip1, which would then lead to ubiquitination and trafficking into EVs. They found that without the WW tag, Cre recombinase was not exported into EVs, and overexpression of WW-Cre recombinase in LN18 cells led to significant expression in EVs, but only when Ndfibp1 was expressed. Furthermore, they discovered that this loaded protein was able to retain its functional ability to induce DNA recombination. These findings suggest that Ndfip1 can be used as a key regulator for extracellular protein loading.

Loading of small molecules

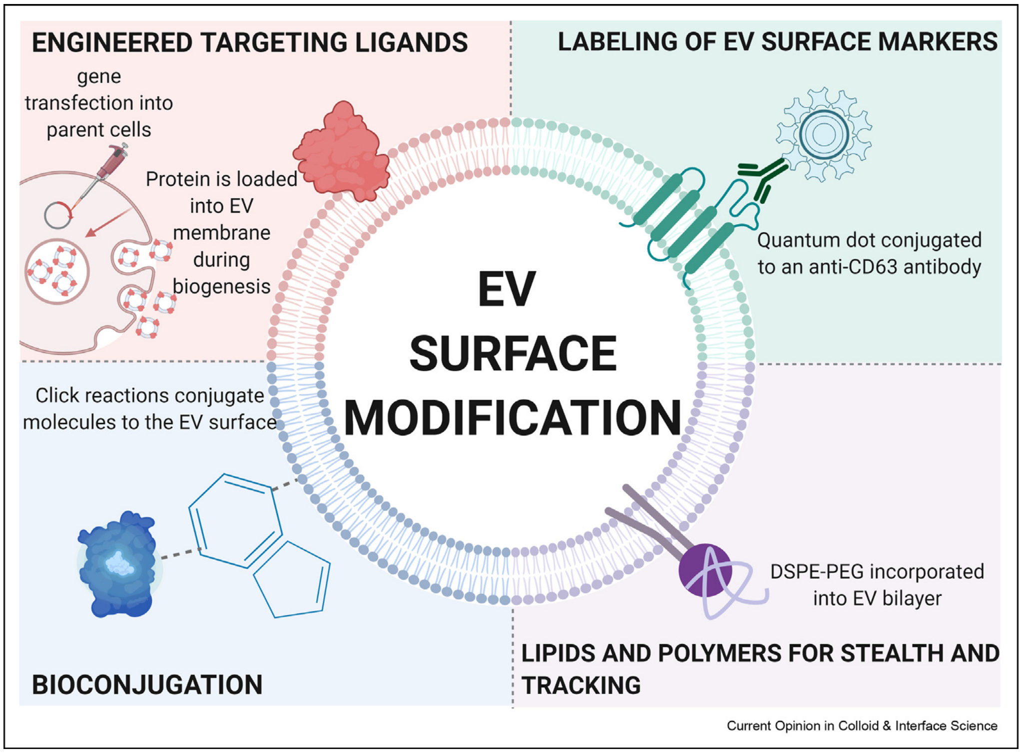
A wide range of small molecules, both hydrophilic and hydrophobic, have been loaded into EVs for therapeutic delivery, which can lead to enhanced targeting, cellular uptake, and stability [23]. Hydrophobic small molecules, such as steroids, polyphenols, are often simply incubated with EVs for loading into the lipophilic portion of the membrane. Gong et al. used macrophage-derived EVs as delivery vehicles for the delivery of hydrophobic doxorubicin (DOX). By passively loading DOX with triethylamine, they were able to achieve maximal loading of ∼160 ng Dox in 1 μg of EVs. They saw enhanced stability and tumor targeting of the drug, which translated into improved anticancer effects in a triple-negative breast cancer model [23]. Studies have also found that active loading methods can be used to further enhance the encapsulation of hydrophobic small molecules, such as paclitaxel (PTX). Kim et al. compared the efficiencies of loading PTX into macrophage-derived EVs using a variety of methods. When comparing incubation at room temperature, electroporation, and sonication, they found that sonication was the most efficient, achieving 28.29% PTX loading [24]. Hydrophilic molecules typically require active loading into the aqueous vesicle core. Fuhrmann et al. tested encapsulation efficiencies of porphyrins with a range of hydrophobicities using both passive and active loading methods. They found that hydrophilic phototoxic porphyrins could be loaded 11-fold higher using saponin-mediate permeabilization when compared to passive incubation. This increase in encapsulation resulted in a >60% increase in cellular uptake of the drug and phototoxicity in triple-negative breast cancer cells [25]. Therefore, it is clear that EVs can be used for the delivery of a wide range of therapeutic small molecules.

Surface decoration for targeting, enhanced circulation, and imaging

An optimized drug delivery system should be able to target cells efficiently, evade immune cell clearance, circulate long-term, and be able to be monitored and tracked easily. Although endogenous EVs can have many of these factors, the surface modification of EVs allows researchers to modulate and/or increase their therapeutic efficacy (Figure 1).

The addition of targeting ligands to the vehicle surface can be engineered through various techniques. The most common method includes gene transfection into the parent cells. Proteins can be overexpressed in the cell and therefore loaded into the EV membrane during biogenesis (Figure 1). Alternatively, fusion proteins with membrane surface proteins can be transfected for surface expression of peptides and proteins that mediate efficient tissue targeting. For example, Wang et al. used a lamp2b fusion protein with the ischemic myocardium targeting peptide CSTSMLKAC to enhance EV targeting directly to an infarct area. They found that 90% of cells were successfully transfected, producing EVs that could target the ischemic myocardium approximately twice as efficiently as the control [26]. By

Figure 1



Major categories for EV Surface modifications. The surface of EVs can be modified to achieve four major outcomes: increased targeting, labeling, bioconjugation, and circulation stealth and tracking.

utilizing targeting moieties, EVs can achieve enhanced retention, tissue distribution, and therapeutic potency.

Another commonly used method for EV surface loading is the binding of EV surface markers. Tetraspanins such as CD63, CD81, CD82, and CD9 are highly expressed on EVs from many different cell types, making them surface modification targets for a variety of purposes. One major way these markers are utilized is through the binding or conjugation of fluorescent reporters so that they can be tracked to shed light on their distribution and mechanisms of action. Sung et al. used a lentiviral vector containing an optimized pHluorin-CD63 construct. pHlourin is a pH-sensitive green fluorescent protein widely used for protein sensing. When attached to CD63, the fusion protein expresses on the surface and fluoresces at neutral pH to track EV secretion [27]. They found that their fusion protein colocalized well with other traditional EV markers and proteins associated with EV biogenesis proteins. They later used this fluorescent signal to confirm that secreted EVs can promote the migration of cancer cells.

In other studies, Dobhal et al. functionalized semiconductive indium quantum dots with a mercaptosuccinic acid (MSA) ligand, which contains a carboxylic acid functional group. This group binds with amino groups present on the CD63 antibody to form a stable carbodiimide linkage. They then showed that this quantum dot/anti-CD63 construct could bind to the surface of monocyte-derived EVs with high efficiency. Through this binding, they could easily image the vesicles and observed a 10-fold increase in EV SPR signal over antibody alone [28]. By utilizing overabundant surface markers, researchers can simplify and optimize their surface loading.

Click chemistry has been utilized as a simple and rapid method for establishing high yield and stable EV surface changes. Click chemistry can be employed for a variety of functional groups under simple reaction conditions, including in the presence of water and oxygen. Song et al. utilized a one-step method for glycoengineering and click chemistry to create EVs labeled with DBCO-Cy5. This was achieved by first treating the parent cells with Ac4ManNAz so they would express azide groups on their surface. The cycloalkyne DBCO then reacts with these azides via cycloaddition to form a stable triazole in one step. They found that this conjugation resulted in high biocompatibility and stability *in vivo* and increased brightness over traditional lipid membrane dyes [29].

Table 1

Applications for EV Surface Modifications. EVs can be decorated with proteins, fluorophores, and lipids for enhanced targeting, tracking, and biodistribution.

EV Source	Method	Application	Reference
Bone Marrow Stromal Cells (BMSCs)	Fusion Protein of Lamp2b and ischemic targeting peptide	Targeting	[28]
HT1080 fibrosarcoma cells, MDA-MB-231 breast cancer cells, and HNSCC61 head and neck squamous carcinoma cells	Fusion protein of pHluorin and CD63	Fluorescent tracking	[71]
THP-1 monocytes	CD63 conjugated Quantum Dots	Florescent tracking	[29]
A549 carcinoma cells	Click Chemistry of (DBCO-Cy5) and EV surface proteins	Fluorescent tracking	[33]
HepG2	Cholesterol lipid anchor for DNA oligos	Detection and quantification	[36]
Bovine Serum	DSPE-PEG lipid Anchor for Mannose	Immune cell evasion and Elevated cellular uptake	[34]

Lipids and polymers can be inserted into EV lipid membranes for increased circulation, targeting, and tracking (Table 1). Electrostatic interactions between cationic lipids and the negatively charged EV membrane have been utilized for surface anchoring [30,31]. He et al. utilized a cholesterol lipid as a membrane anchor for the loading of horseradish peroxidase (HRP) for enhanced EV detection. First, custom oligonucleotide sequences conjugated with terminal cholesterol and biotin moieties were purchased. Complementary sequences were then hybridized, and the cholesterol portions of the construct were inserted into the EV lipid membrane through hydrophobic interactions, leaving a hydrophilic sticky end of DNA exposed at the surface. Further, enzyme-linked DNA hybridization chain reaction (HCR) was used to amplify the signal, and streptavidin-conjugated HRP, when bound to the biotin moieties, could be used for detection. Ultimately, they concluded that EVs could be passively loaded with cholesterol with high efficiency, and this could be used for enhanced EV detection [32]. Choi et al. utilized DSPE-PEG to load the surface of bovine serum-derived EVs with mannose for the stimulation of mannose receptors on the surface of dendritic cells. First, they discovered that the simple physical incorporation of DSPE-PEG into the EV bilayer was significantly more efficient than the chemical incorporation of NHS-PEG, demonstrating 70% vs. 40% loading efficiency, respectively. Although traditionally incorporating PEG into the lipid membrane decreases nonspecific cellular uptake and enhances immune cell evasion, the addition of mannose to DSPE-PEG increased the dendritic cell uptake 2.1-fold [33].

With these surface modifications, it is clear to see that EVs can serve as enhanced systems for drug delivery and cellular communication.

EV production: boosting biogenesis

EV yield remains one of the main limitations of using EVs for therapeutic applications. Previous strategies for increasing EV biogenesis include inducing hypoxia, overexpressing tetraspanin CD9 [34], increasing the amount of Ca^{2+} ionophores, and preconditioning cells with thrombin [35]. Although the mechanisms behind these methods are not completely understood, studies have shown that they primarily affect proteins and lipids that mediate EV release. Gonzalez-King et al. found that inducing hypoxia increases hypoxia-inducible factor-1α (HIF-1α), which is associated with the Rab protein Rab22A that helps with the formation of microvesicles [35]. HIF-1α also modulates SMPD3 expression, a lipid metabolic enzyme that plays a role in early EV formation by generating ceramide, which triggers EV budding in multivesicular bodies [34].

Table 2

Conditions for boosted EV production. Studies have shown that EV secretion or biogenesis can be increased through increased expression of specific proteins and lipids, as well as optimized culture conditions.

Method	Mechanism	Boost in EV Production	Effect on EV composition	Reference
Norepinephrine and N-methyldopamine,	Increased expression of SMPD3 and ceramide	~3 -fold	0.3% of total proteins downregulated, 0.6% of total proteins upregulated	[34]
MOPIPP and Vacuolin-1	Interference of endosomal trafficking to lysosome	>3 fold	No change in miRNA	[36]
Sodium iodoacetate and 2,4-dinitrophenol (IAA/DNP)	Release of adenosine and 2′,3′-cAMP and increase in ceramide synthesis	≥10-fold	Not examined	[37]
Cellular Nanoporation	Increase of heat shock protein expression	50-fold	Endogenous components not examined	[38]
BioNOC II microcarrier	Increased cell growth density	$\sim 1.2 \times 10^8 \pm 0.56 \times 108$ p/mL	Not examined	[40]
3D cell culture with tangential flow	Biomimetic 3D cultures	140-fold	No significant changes in protein expression	[41]
Hypoxia	Increased expression of Rab22A	>2 fold	Increase in proteins associated with HIF-1a stabilization and metabolic processes; overexpression of many miRNAs	[35]

Recent studies have identified small molecules that affect SMPD3 and ceramide expression and, in turn, increase EV production. Our group selected five different compounds − fenoterol, norepinephrine, N-methyldopamine, mephenesin, and forskolin − from a screening of repositioned drugs that have shown to increase EV production in prostate cancer cells and tested them on mesenchymal stem cells. We were able to increase EV production by up to ∼3-fold by combining norepinephrine with N-methyldopamine [34]. An increase in SMPD3 and endosomal Rab27a and Rab27b, key proteins in EV biogenesis and secretion, was also detected, confirming the association of these proteins with EV formation and secretion. We found that these profound changes in production did not alter EV composition, though. After treatment, only 0.3% and 0.6% of proteins were statistically downregulated or upregulated, respectively. Li et al. explored the use of two small molecules, MOPIPP and vacuolin-1, on the stimulation of EV secretion from 293T and U251 cells. They hypothesized that these molecules could promote vacuolization in late endocytic compartments and disrupt the trafficking of late endosomes to lysosomes, leading to EV escape to the plasma membrane. They observed that there was a significant increase in vacuolization and a >3-fold increase in EV markers, ALIX, CD63, and LAMP-1 from isolated vesicles [36]. These results suggest that EV release also increased at least several-fold. Furthermore, they found that for six representative miRNAs, the small molecule induced EVs expression had no significant changes besides slightly increased expression [36]. Overall, the results from these studies suggest that boosting EV biogenesis does not necessarily lead to a change in vesicle composition. The approach of using small molecules to modulate EV production could be advantageous as it could be used under *in vitro* and *in vivo* conditions.

Ludwig et al. took a different approach to increase EV production, using sodium iodoacetate and 2,4-dinitrophenol (IAA/DNP). When IAA/DNP was released, it led to inhibition of glycolysis and oxidative phosphorylation in the cells. This resulted in a depletion of energy, which caused the cells to release adenosine and 2′,3′-cAMP, a stimulator of EV release. After 48 h, EV secretion increased ≥10-fold. They were able to further stimulate EV production with an inhibitor of AMP-activated protein kinase, dorsomorphin [37]. Last, cellular nanoporation was used in another study to increase EV release by increasing Ca^{2+} in the extracellular space and increasing the expression of heat shock proteins (HSP) [38]. These HSPs regulate P53 activity, which is a gene that regulates EV production through TSAP6, a protein that colocalizes with the trans-Golgi network responsible for EV biogenesis [39]. The Ca^{2+} also increased EV production through the aforementioned calcium-dependent mechanism. Using this method, 50-fold more EVs were produced [38]. It is unclear whether this method of boosting EV secretion can only be achieved through cellular nanoporation or if other cell stress-inducing strategies can trigger the same response. However, cellular nanoporation still proves to be an effective method for increasing EV secretion.

In addition to these findings, recent studies have explored new manufacturing processes and tools for mass EV production. Current large-scale production methods involve large cell culture flasks and multiple bioreactors that are multidimensional and multilayered to increase surface area and cell density. The most significant increases in EV production have occurred in studies that utilize 3D cell culture. Some of the challenges that come with these current methods include the cost of culture expansion and changes in the phenotype of the large-scale culture that affects EV function [40]. One method for large-scale EV production used fiber-based microcarriers called BioNOC II12 [40] to provide a support matrix and additional surface area for cell attachment. As a result, $\sim 1.2 \times 10^8 \pm 0.56 \times 10^8$ particles/mL were produced in carrier-based culture, compared to the $\sim 0.27 \times 10^8 \pm 0.06 \times 10^8$ particles/mL of EVs produced in the traditional cell culture. Other benefits of this method were increased time efficiency, lower cost due to less medium and plastic material used, and no changes in EV characteristics. A 2018 study incorporating the 3D cell culture of umbilical cord-derived mesenchymal stem cells and tangential flow filtration produced 140-fold amounts of EVs compared with the conventional 2D culture with ultracentrifugation [41]. A 2019 study by Patel et al. showed that a 3D perfusion culture of endothelial cells could be used to increase EV production by more than 100-fold. They also showed that previous methods, using ethanol conditioning to enhance endothelial cell vascularization function, could be incorporated into the dynamic culture as well. They demonstrated that the secreted EVs had boosted expression levels of characteristic angiogenesis markers [42]. However, they also observed that the total protein content per EV was reduced approximately 15-fold (Table 2).

Studies have also sought to boost EV production through the selection of specific parent cells. Mesenchymal stem cells are a very popular source for EV production because they produce high EV yield and are isolated from noncontroversial human materials [43]. An engineered human amniocyte cell line called CAP cells (CEVEC's amniocyte production) has also been identified as an effective source for EV production due to their ability to be preloaded with therapeutic miRNAs through miRNA overexpression [43].

EV storage

A critical barrier for the use of EVs and other EVs as drug delivery vehicles in therapeutic applications is the limited knowledge of their optimal storage conditions. The ability for stored EVs to be stable and easily recoverable would greatly enhance their applicability in the field.

Initial studies have shown that EVs are typically more stable in colder temperatures; however, there is still much discussion about how stable EVs are after freezing. Recent studies have continued to optimize storage conditions to combat any changes in membrane stability (Table 3). For example, Cheng et al. explored the combinatory effects of pH, temperature, and freeze-thawing on EV stability and downstream function. Using Nanoparticle Tracking Analysis (NTA) and Western blotting to determine EV concentration and representative protein concentrations, respectively, they found that the acidic environment of pH 4 decreased EV concentration by more than 60% and decreased the ALIX, HSP70, and TSG101 protein concentrations by approximately 50% when compared to EVs stored at neutral pH, showing a loss in EV stability and recovery. They also found that with an increasing number of freeze—thaw cycles, concentrations and protein levels also decreased, signaling EV degradation [44]. Additionally, they concluded that EVs stored below −70 °C had the best recovery. They also found that EVs that had been stored at 4 °C, freeze-thawed four times or more, or stored at pH 7 had the lowest cellular uptake. These results show just how much variability can be created with each conditional change.

Lyophilization presents an attractive option for EV storage due to the possibility of extended shelf life and ease of transportation and handling. Charoenviriyakul et al. sought to compare the differences in stability between B16BL6 melanoma-derived EV, which were lyophilized and stored at room temperature and those frozen at −80 °C for one week. TEM images showed that the lyophilized EVs formed aggregates and had a much wider polydispersity index (PDI). However, upon the addition of the cryoprotectant, trehalose, the aggregate formation was attenuated, and the PDI was comparable to EVs stored at −80 °C. This is significant because trehalose, a natural, nontoxic sugar, has been commonly used as a protein stabilizer and has already been used in FDA approved therapeutic formulations. Additionally, they observed that lyophilized B16BL6 vesicles maintained their proliferation activity. Next, they found that lyophilized EVs loaded with a functional nucleic acid, CpG, could stimulate dendritic cells to release more TNF-α and IL-6 than untreated cells or

Table 3

Optimal Conditions for EV Storage. EV storage and stability are primarily impacted by temperature, buffer composition, mechanical manipulation.

Factors Tested	Optimal Conditions	Method For EV Stability Testing	Tested Storage Time	Reference
pH, temperature, and freeze thaw	pH 4, −70 °C, and <4 freeze thaw cycles	NTA and Western blot	24 h	[44]
Cryoprotectant, freeze thaw cycles, and cryoprotectant	−80 °C in 25 mM trehalose	Electron tomography, macrophage immune assays (function), NTA, Bradford assay, immunoblotting, zeta potential	1 month	[45]
Lyophilization and cryoprotectant	Lyophilization with100 mM trehalose and 5% PVP	NTA, Western blot. TEM, viability and proliferation studies	Unknown	[46]
Lyophilization and cryoprotectant	Ultrafiltration and lyophilization with 0.5% mannitol	BCA, Nile red assay, cytokine ELISA, NTA, FTIR, evaluation of proliferation and cytokine secretion	Unknown	[47]
Bovine vs. human milk extracellular vesicles	Bovine milk stored at 4 °C	qRT-PCR, miRNA sequencing	4 weeks	[49]

CpG alone. Last, they tested if the proteins loaded into EVs maintained function after lyophilization. They found that the luciferase in lyophilized EVs maintained activity when compared to EVs stored at −80 °C [45].

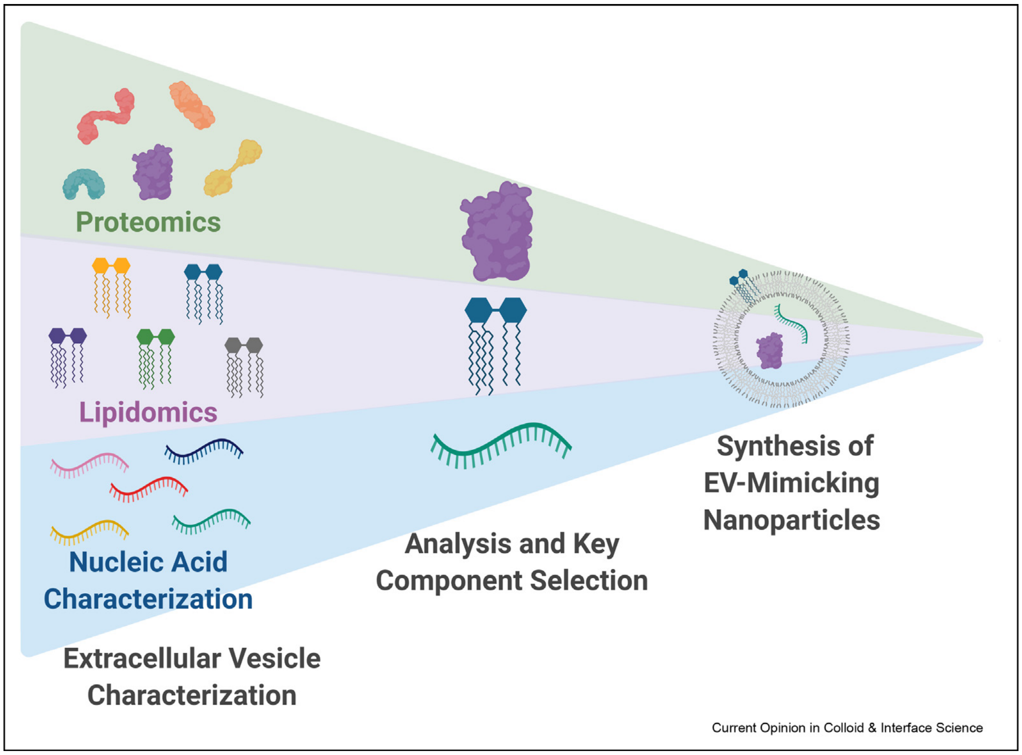
Studies have found that the isolation procedures before storage are also major contributors to long-term stability. Baradie et al. compared the use of ultrafiltration (UF) and ultracentrifugation (UC) isolation methods and the use of two different lyoprotectants in conjunction with lyophilization to determine the effects on stem cell EV stability. They found that ultrafiltration yields three-fold more particles than ultracentrifugation and that lyophilization alone greatly reduced the number of EV particles. However, upon the combination of ultrafiltration and lyophilization with the use of trehalose and/or polyvinylpyrrolidone (PVP40), the concentration and morphology of EVs could be conserved [46]. They observed that EVs isolated by ultrafiltration and then tangential fluid filtration and then freeze-dried with trehalose could significantly increase the viability of ischemic myoblasts ($p < 0.0385$). Additionally, Bari et al. explored whether UF or UC and lyophilization with mannitol as a cryoprotectant could retain stem cell-derived EV membrane integrity and function. They found that ultrafiltration led to higher concentrations of EVs and their endogenous proteins, lipids, and cytokines. By retaining these bioactive molecules, these EVs could be used for immunomodulation, as shown by a dose-dependent proliferation of phytohemagglutinin (PHA)-activated peripheral blood mononuclear cell (PBMCs) [47]. Please refer to the review by Jeyaram et al. for a detailed summary of storage conditions and their effects on EV stability before 2017 [48].

Despite these advances in overall EV storage stability, other researchers have turned to alternative EV sources and storage conditions for storage optimization. For example, Leiferman et al. explored the storage stability of EVs in human breast milk. They found that when human milk was stored at 4 °C for four weeks, the recovery of EVs progressively dropped to almost 50%, though no significant changes were seen when the milk was frozen or stored with preservatives. Additionally, they found that long-term storage of bovine milk did not have the same degradative effect on EVs as human milk, as the count and size of the vesicles were maintained for at least four weeks [49]. There was no clear explanation as to why this was. This is significant because it further confirms that the EV source and storage solutions can make large impacts on long term storage, although they may need to be tested on a case-by-case basis.

Reverse engineering

EVs, with their promising therapeutic potentials, have also been implicated in various pathogenic pathways [50]. To circumvent these challenges and achieve the

Figure 2



Pipeline for Reverse Engineering Artificial Extracellular vesicles. For reverse engineering, EVs need to be first characterized by their protein, lipid, and nucleic acid content. Once key factors are identified, they can be synthesized and incorporated independently in lipid vesicles to create EV mimicking nanoparticles with defined and optimized EV composition and function.

desired effects, some researchers have taken a “bottom-up” approach, isolating only the most potent and beneficial factors of endogenous EVs to create optimized synthetic nanoparticles. For doing this, EVs from distinct subsets must be characterized by their three primary components: nucleic acids, proteins, and lipids. By understanding the key players in each of these factors, researchers can create custom EVs or EV-like vehicles that are fine-tuned for various disease treatments (Figure 2).

Nucleic acids, particularly mRNAs and miRNAs, have frequently been identified as the primary mediators of EV therapeutic functions [51,52]. However, studies have shown that other noncoding RNA, such as viral RNAs, ribosomal RNAs, and transfer RNA, may be key players in EV action. Thus, there is an increasing need to identify the total nucleic acid content of EV subpopulations. Furthermore, findings from large-scale screening studies have been compiled into databases for those wanting to quickly identify potential therapeutic agents and targets.

Using these methods, EVs derived from therapeutically active parent cells, such as mesenchymal stem cells and immune cells, have been particular points of interest [53,54]. Researchers have identified that these cell types are highly enriched with miRNAs that are largely associated with proliferation, angiogenesis, and immunomodulation pathways. For example, Ferguson et al. used miRNA profiling to characterize mesenchymal stem cell-derived EVs. They found that 23 miRNA sequences accounted for approximately 80% of the total RNA content. Furthermore, many of these miRNAs targeted the same group of genes involved in the TGF-β pathway. To confirm the roles of these nucleic acids, a representative miRNA, miR199a, was loaded into MSC EVs, and markers for cardiomyocyte proliferation and apoptosis were assessed. When compared to unmodified EVs, miR199a EVs downregulated apoptosis marker expression by approximately 50% and significantly increased proliferation in MSCs at EV doses of 2 μg [55].

Proteins are perhaps the most widely studied component of EV composition due to their impacts on biological function through targeting. While it is accepted that EV proteins are key mediators of cellular binding and uptake, understanding the immunomodulatory effects of EVs proteins is equally important. Proteomic analysis of immune cell-derived EVs has confirmed that these vehicles contain many of the same proteins as the parent cells and are capable of immunomodulation. Dendritic cell and B cell-derived EVs contain MHC receptors on their cell surface and can serve as antigen-presenting vesicles for T cell priming and activation [56,57]. These findings are particularly important as they mean EVs may be one of the primary factors during an endogenous immune response. However, their mechanisms of action are not fully known. Lu et al. observed that EVs derived from activated CD4+ T cells had high levels of CD40L, which is important for the activation, proliferation, and antibody production of B cells [58]. Recent studies have shown early success in using immune cell-derived EVs to modulate the immune response in various disease models. For example, Fu et al. showed that EVs released by CAR-T cells contain many cytotoxic components and can inhibit tumor growth. They observed that these EVs, unlike their parent cells, do not express PD1, which means they cannot be adversely affected by PDL1, a major barrier in current CAR-T therapies. Additionally, they found that during *in vivo* studies, CAR-T EVS were just as safe as the established cell therapies [59]. These studies suggest that immune cell EVs may be viable replacements for live-cell therapies, reducing possible risk while maintaining potency.

While not as widely characterized as nucleic acids or proteins, EV lipids, which are largely expressed in the membrane, have also been shown to affect cellular uptake, membrane trafficking, and cell-to-cell communication [60]. A 2016 study by Haraszti et al. examined the lipidome of three cell types: U87 glioblastoma cells, Huh7 hepatocellular carcinoma cells, and human bone marrow-derived mesenchymal stem cells (MSCs). They found that EVs from the three cell types were enriched in glycolipids, free fatty acids, and phosphatidylserines. Moreover, Huh7 and MSC EVs were specifically enriched in cardiolipins, while U87 EVs were enriched in sphingomyelins [61].

Despite the increasing knowledge of EV composition and biological involvement, few studies have used this information to create artificial EVs for drug delivery. Haraszti et al. used lipidomic and proteomic analysis to identify three key proteins and one lipid (Rab7, Desmoplakin, AHSG, and dilysocardiolipin) that were overexpressed in stressed serum-deprived mesenchymal stem cell EVs. By combining these factors, they were able to create artificial EVs that were just as effective as endogenous stressed EVs at delivering siRNA to neurons, a 22-fold improvement over EVs from control cells [62]. Zhang et al. focused solely on utilizing the expression of a few key proteins. They hypothesized that therapeutic membrane proteins from multiple cell sources could be combined and inserted into a synthetic lipid vesicle. Knowing that red blood cells (RBCs) overexpress CD47 to escape phagocytosis and that MCF-7 cancer cells overexpress proteins that allow homing to other cancer cells, they further hypothesized that EV-mimicking vesicles that contain both types of these proteins could target tumor sites with extended circulation time. Using Western blot analysis, they found that their artificial hybrid EV contained similar levels of CD47 to endogenous RBCs and similar expression levels of EPCAM, Galectin 3, and N Cadherin to endogenous MCF-7 cells. Furthermore, these chimeric EVs exhibited significantly enhanced internalization in MCF-7 cells and macrophage escape, which the authors contributed to the membrane proteins [63].

While seemingly effective, the major pitfall of these approaches is that they require extensive characterization of endogenous vesicle subpopulations before synthetic particle formation. Studies have shown that every subpopulation is unique and must be analyzed thoroughly [7,60]. This process can be somewhat simplified by solely relying on previously published data; however, protein expressions from the same cell line can differ from study to study, making it difficult to identify reliable targets. For expanding upon these studies, reproducibility must be tested.

Clinical trials - the latest status of the field

Although there has been an expansive pursuit of EV-based therapeutics in preclinical settings, this has not yet translated to clinical output. Furthermore, within the limited number of current clinical trials, even fewer utilize EVs as drug delivery systems. Instead, most studies collect these vesicles as biomarkers for disease or secondary endpoints for treatments. Major limitations in their clinical advancements include the lack of standardized GMP practices and scalability. For expanding their applicability, clinical trials must work to first establish their safety and toxicity, pharmacokinetics, and various mechanisms of action. Below in Table 4, we outline the completed and active clinical trials using EV as delivery vehicles. Although not all of these results have been reported, these trials provide the foundation for future studies utilizing modified EVs for optimized effects.

Mesenchymal stem cells are the clear focus of current clinical trials for EV therapeutics. This is likely due to their well-established angiogenic, regenerative, and immunomodulatory effects [64,65]. Their ability to self-renew and differentiate into a variety of cell types makes them particularly useful. Additionally, MSCs have been shown to naturally home to sites of inflammation, making them ideal for wound healing and tissue repair [66]. Moreover, it is thought that much of their therapeutic action is mediated by the release of their EVs [67]. Their potential benefits and limited adverse effects make them a logical first choice for evaluating EVs in a clinical setting. The clinical trials highlighted here

Table 4

Completed or active clinical trials using extracellular vesicles as drug delivery vehicles. Source: Clinicaltrials.gov.

ID	Cell Source	Indication	Administration	Primary Endpoints	Status
NCT04389385	COVID-19 specific T cells	Corona Virus Infection Pneumonia	Inhalation	Adverse reactions, efficacy assessment, and the rate of recovery without a mechanical ventilator	Active, not recruiting
NCT01668849	Grape Cells	Head and Neck Cancer Oral Mucositis	Oral	Pain caused by oral mucositis	Active, not recruiting
NCT01294072	Plants	Colon Cancer	Oral	Concentration of curcumin in normal and cancerous tissue	Active, not recruiting
NCT04491240	MSCs	COVID-19 SARS-CoV-2 PNEUMONIA	Inhalation	Number of subjects with treatment-emergent adverse events during the post-treatment phase	Completed, has results
NCT04281901	Plasma	Otitis Media Chronic Temporal Bone	Platelet and EV rich-plasma soaked ear wicks	Change in inflammation surface area and change in chronic otitis media questionnaire	Completed, has results
NCT04276987	MSCs	Coronavirus	Inhalation	Adverse reaction (AE) and severe adverse reaction (SAE) and time to clinical improvement (TTIC)	Completed
NCT04134676	Wharton's Jelly MSCs	Chronic Ulcer	Topical	Knowing the success rate of chronic ulcer healing in patients undergoing wound care with conditioned medium	Completed
NCT02594345	Red Blood Cell Units	Healthy Volunteers	*In vitro* mixing	Clotting time	Completed
NCT01159288	Dendritic Cells	Nonsmall Cell Lung Cancer	Intradermal	Progression-free survival Cancer vaccine	Completed

cover various administration routes and indications, with many centered around inflammation and damage. In a phase II clinical trial, NCT01159288, dendritic cell-derived EVs (Dex) loaded with cancer antigens EVs were used as a vaccine for nonsmall cell lung cancer. They observed that 32% of patients exhibited progression-free survival four months after chemotherapy cessation and that Dex treatment boosted Natural Killer antitumor immunity [68]. In a recent study, NCT04491240, they found that inhalation of MSC in COVID19 patients led to no adverse effects and led to a statistically significant decrease in C reactive protein and LDH levels, which are both signs of damage and inflammation. In another study, NCT04281901, patients with chronic postoperative temporal bone inflammation were treated with ear wicks soaked in platelet and EV rich plasma (PVRP). They observed a 64% reduction in the inflammation surface area for PVRP treated patients, but a 50% increase for standardly treated patients. Together, these studies provide a promising basis for therapeutic EV use, especially for inflammatory indications, and as more is understood about the biological roles of EVs, their progress in clinical trials will continue to advance.

Conclusions and proposed future directions

It is becoming ever evident that EVs are complex biomaterials that can play a variety of roles in therapeutic applications. We propose that the following developments are necessary to fully utilize the potential of EVs as drug delivery vehicles. First, standardized methods for characterizing a wide range of EV subpopulations are desperately needed for the three primary components defined in Figure 2. While many studies focus primarily on characteristic proteins and nucleic acids as markers for EV function and stability, this disregards all other key factors that may play a role in the mechanism of EV action. EVs need to be characterized by their protein, nucleic acid, and lipid content. This is particularly true for lipid composition, which is often disregarded during vehicle characterization but has been shown to mediate EV function [6]. While databases exist that compile findings of previous studies, it is difficult to draw conclusions between studies. Practices need to be standardized so that it can be understood how EV composition differs from cell type to cell type and to examine the heterogeneity that lies within the same subpopulations.

Second, there should be an increased push to explore the use of EVs from unconventional sources such as bacteria and fungi. Bacteria and fungi have not been deeply explored for therapeutic applications, likely due to their potential for immunogenicity and adverse effects. The limited studies that do utilize these bacteria focus on their use as immunomodulatory adjuvants or vaccines, but even fewer have focused on their use as drug delivery vehicles [69]. Bacteria and fungi are regularly used for protein and metabolite production due to their ability to be scaled greatly and their human orthologs. However, this has not translated to their secretion of EVs. Gram-positive bacteria can be a potential focus for this process due to their single membrane compositions and their lack of toxic lipopolysaccharides [70]. Furthermore, studies have shown that microbial membrane vesicles can accumulate at the tumor site and activate antitumor cascades [71]. By modifying these cell lines to further reduce potentially toxic components, they could serve as potent agents. Immunogenic components can still be utilized to create vehicles that can serve as both therapeutic carriers and immune interventions. While there is much to do in the way of modifying these vehicles to ensure safety, by utilizing these cell sources, EVs may be scaled effectively for potential clinical use.

Third, an important step for employing EVs in a clinical setting is the ability to efficiently produce them on a large scale. However, increased production may lead to compositional changes and, therefore, functional changes. Very few studies look beyond expression levels of membrane proteins or nano tracking analysis. As previous studies have shown, EV components such as total RNA or protein can be altered after a boost in EV secretion, which can alter cellular uptake, immunomodulatory effects, and more. Therefore, there is an important need to characterize EV composition holistically after scaling up. EVs should be examined using a combination of analytical methods, such as flow cytometry and mass spectrometry, and biological assays.

Fourth, while many studies have focused on optimal methods for EV storage stability, many studies do not consider what happens to their biological function after storage. For ensuring reproducible studies that will translate clinically, there is an urgent need to take this analysis a step further to ensure that morphological or colloidal stability aligns with the maintenance of biological activity.

Fifth, reverse engineering provides a mechanistic approach to understand EV. The capacity to utilize EVs effectively as therapeutic delivery vehicles is limited by their component complexity. Despite the numerous studies on EV efficacy, many of their mechanisms of action are poorly understood. For addressing these challenges, it is important to examine EV composition through a reductionist approach. By isolating individual factors and understanding their effects on EV function, we can begin to incorporate only the components that contribute to our desired effects. Heterogeneity between EV subsets creates a complex picture that can complicate their use. Therefore, in-depth nucleic acid, proteomic, and lipidomic analyses are necessary to fully characterize each EV population. This methodical approach will not only shed light on how EVs act endogenously but how they can be utilized as nanocarriers. Future studies will improve upon the creation of synthetic EVs or EV-mimics that are fine-tuned for potent drug delivery.

By addressing these challenges, researchers can create optimized delivery vehicles using EVs. These vesicles can be utilized by harnessing factors that allow them to carry endogenous cargo and applying them towards therapeutic agents of interest. This can include a wide variety of small molecules, nucleic acids, proteins, and lipids. Furthermore, their use in drug delivery can be enhanced with the use of selective surface decoration to improve circulation, targeting, tracking, and biodistribution.

In conclusion, while unmodified EVs have shown great therapeutic potential on their own, the ability to extensively modify their physicochemical properties and features makes them attractive candidates for novel delivery approaches. Here we have overviewed current techniques for optimizing EV cargo loading, surface modification, production, storage, and methods for artificial EV synthesis. By incorporating optimized strategies across each of these factors, we hope that researchers can create EVs that can be loaded efficiently, labeled or targeted, and produced and stored in large quantities. While much of this optimization is EV subtype specific, creating a multifaceted approach for drug delivery will help us to further understand EV function and improve their clinical potential.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References
Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest

1. Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C: **Exosomal-like vesicles are present in human blood plasma**. *Int Immunol* 2005, **17**:879–887, https://doi.org/10.1093/intimm/dxh267.

2. Rutter BD, Innes RW: **Extracellular vesicles isolated from the leaf apoplast carry stress-response proteins**. *Plant Physiol* 2017, **173**:728–741, https://doi.org/10.1104/pp.16.01253.

3. Munagala R, Aqil F, Jeyabalan J, Gupta RC: **Bovine milk-derived exosomes for drug delivery**. *Canc Lett* 2016, **371**: 48–61, https://doi.org/10.1016/j.canlet.2015.10.020.

4. de Broe ME, Wieme RJ, Logghe GN, Roels F: **Spontaneous shedding of plasma membrane fragments by human cells in vivo and in vitro**. *Clin Chim Acta* 1977, **81**:237–245, https://doi.org/10.1016/0009-8981(77)90054-7.

5. Lotvall J, Valadi H: **Cell to cell signalling via exosomes through esRNA**. *Cell Adhes Migrat* 2007, **1**:156–158, https://doi.org/10.4161/cam.1.3.5114.

6. Ferguson SW, Nguyen J: **Exosomes as therapeutics: the implications of molecular composition and exosomal heterogeneity**. *J Contr Release* 2016, **228**:179–190, https://doi.org/10.1016/j.jconrel.2016.02.037.

7. Ferguson S, Kim S, Lee C, Deci M, Nguyen J: **The phenotypic effects of exosomes secreted from distinct cellular sources: a comparative study based on miRNA composition**. *AAPS J* 2018, **20**:67, https://doi.org/10.1208/s12248-018-0227-4.

8. Abd Elmageed ZY, Yang Y, Thomas R, Ranjan M, Mondal D, Moroz K, *et al.*: **Neoplastic reprogramming of patient-derived adipose stem cells by prostate cancer cell-associated exosomes**. *Stem cell* 2014, **32**:983–997, https://doi.org/10.1002/stem.1619.

9. Yamashita T, Takahashi Y, Takakura Y. *Possibility of exosome-based therapeutics and challenges in production of exosomes eligible for therapeutic application*, vol. 41; 2018.

10. Nguyen J, Szoka FC: **Nucleic acid delivery: the missing pieces of the puzzle?** *Acc Chem Res* 2012, **45**:1153–1162, https://doi.org/10.1021/ar3000162.

11. Ferguson SW, Megna JS, Nguyen J: **3 - composition, physicochemical and biological properties of exosomes secreted from cancer cells**. In *Diagnostic and Therapeutic Applications of Exosomes in Cancer*. Edited by Amiji M, Ramesh RBT, Academic Press; 2018:27–57, https://doi.org/10.1016/B978-0-12-812774-2.00003-1.

12. Wang J, Lee CJ, Deci MB, Jasiewicz N, Verma A, Canty JM, *et al.*: **MiR-101a loaded extracellular nanovesicles as bioactive carriers for cardiac repair**. *Nanomed Nanotechnol Biol Med* 2020, **27**:102201, https://doi.org/10.1016/j.nano.2020.102201.

13. Kooijmans SAA, Stremersch S, Braeckmans K, de Smedt SC, Hendrix A, Wood MJA, *et al.*: **Electroporation-induced siRNA precipitation obscures the efficiency of siRNA loading into extracellular vesicles**. *J Contr Release* 2013, **172**:229–238, https://doi.org/10.1016/j.jconrel.2013.08.014.

14. Sachdev S, Feijoo Moreira S, Keehnen Y, Rems L, Kreutzer MT, Boukany PE: **DNA-membrane complex formation during electroporation is DNA size-dependent**. *Biochim Biophys Acta Biomembr* 2020, **1862**:183089, https://doi.org/10.1016/j.bbamem.2019.183089.

15. Jeyaram A, Lamichhane TN, Wang S, Zou L, Dahal E, Kronstadt SM, *et al.*: **Enhanced loading of functional miRNA cargo via pH gradient modification of extracellular vesicles**. *Mol Ther* 2020, **28**:975–985, https://doi.org/10.1016/j.ymthe.2019.12.007.

16. Villarroya-Beltri C, Gutiérrez-Vázquez C, Sánchez-Cabo F, Pérez-Hernández D, Vázquez J, Martin-Cofreces N, *et al.*: **Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs**. *Nat Commun* 2013, **4**:1–10, https://doi.org/10.1038/ncomms3980.

17. * Temoche-Diaz MM, Shurtleff MJ, Nottingham RM, Yao J, Fadadu RP, Lambowitz AM, *et al.*: *Distinct mechanisms of microRNA sorting into cancer cell-derived extracellular vesicle subtypes*. 2019, https://doi.org/10.7554/eLife.47544.001.

Using biochemical and genetic tools, the authors demonstrate that miR-122 contains motifs that bind to Lupus La protein for selective sorting. This is the first paper to identify this protein as a sorting mediator and this sorting pathway.

18. Yim N, Ryu SW, Choi K, Lee KR, Lee S, Choi H, *et al.*: **Exosome engineering for efficient intracellular delivery of soluble proteins using optically reversible protein-protein interaction module**. *Nat Commun* 2016, **7**:1–9, https://doi.org/10.1038/ncomms12277.

19. Liu C, Su C: **Design strategies and application progress of therapeutic exosomes**. *Theranostics* 2019, **9**:1015–1028, https://doi.org/10.7150/thno.30853.

20. Haney MJ, Klyachko NL, Zhao Y, Gupta R, Plotnikova EG, He Z, *et al.*: **Exosomes as drug delivery vehicles for Parkinson's disease therapy**. *J Contr Release* 2015, **207**:18–30, https://doi.org/10.1016/j.jconrel.2015.03.033.

21. * Sukreet S, Silva BVRE, Adamec J, Cui J, Zempleni J: **Sonication and short-term incubation alter the content of bovine milk exosome cargos and exosome bioavailability (OR26-08-19)**. *Curr Dev Nutr* 2019, **3**, https://doi.org/10.1093/CDN/NZZ033.OR26-08-19.

Using Western blot, the authors demonstrated that sonication resulted in a loss of E protein markers. This study is one of the first to thoroughly examine the effects of sonication on EV biological function.

22. Sterzenbach U, Putz U, Low L-H, Silke J, Tan S-S, Howitt J: *Engineered exosomes as vehicles for biologically active proteins*. 2017, https://doi.org/10.1016/j.ymthe.2017.03.030.

23. Gong C, Tian J, Wang Z, Gao Y, Wu X, Ding X, *et al.*: **Functional exosome-mediated co-delivery of doxorubicin and hydrophobically modified microRNA 159 for triple-negative breast cancer therapy**. *J Nanobiotechnol* 2019, **17**:93, https://doi.org/10.1186/s12951-019-0526-7.

24. Kim MS, Haney MJ, Zhao Y, Mahajan V, Deygen I, Klyachko NL, *et al.*: **Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells**. *Nanomed Nanotechnol Biol Med* 2016, **12**:655–664, https://doi.org/10.1016/j.nano.2015.10.012.

25. Fuhrmann G, Serio A, Mazo M, Nair R, Stevens MM: **Active loading into extracellular vesicles significantly improves the cellular uptake and photodynamic effect of porphyrins**. *J Contr Release* 2015, **205**:35–44, https://doi.org/10.1016/j.jconrel.2014.11.029.

26. Wang X, Chen Y, Zhao Z, Meng Q, Yu Y, Sun J, *et al.*: **Engineered exosomes with ischemic myocardium-targeting peptide for targeted therapy in myocardial infarction**. *J Am Heart Assoc* 2018, **7**, https://doi.org/10.1161/JAHA.118.008737.

27. Sung BH, von Lersner A, Guerrero J, Krystofiak ES, Inman D, Pelletier R, *et al.*: **A live cell reporter of exosome secretion and uptake reveals pathfinding behavior of migrating cells**. *Nat Commun* 2020, **11**:2092, https://doi.org/10.1038/s41467-020-15747-2.

28. Dobhal G, Ayupova D, Laufersky G, Ayed Z, Nann T, Goreham R v: **Cadmium-free quantum dots as fluorescent labels for exosomes**. *Sensors (Switzerland)* 2018, **18**:1–12, https://doi.org/10.3390/s18103308.

29. * Song S, Shim MK, Lim S, Moon Y, Yang S, Kim J, *et al.*: *In situ one-step fluorescence labeling strategy of exosomes via bio-orthogonal click chemistry for real-time exosome tracking in vitro and in vivo*. 2020, https://doi.org/10.1021/acs.bioconjchem.0c00216.

Using click chemistry, the authors demonstrate that EVs can be labeled and tracked with DBCO-Cy5. This study highlights a novel labeling method that outcompetes traditional lipid dyes. This is the first study to utilize an in situ fluorescence labeling method for EVs.

30. Nakase I, Futaki S: **Combined treatment with a pH-sensitive fusogenic peptide and cationic lipids achieves enhanced cytosolic delivery of exosomes**. *Sci Rep* 2015, **5**, https://doi.org/10.1038/srep10112.

31. Sawada SI, Sato YT, Kawasaki R, Yasuoka JI, Mizuta R, Sasaki Y, *et al.*: **Nanogel hybrid assembly for exosome intracellular delivery: effects on endocytosis and fusion by exosome surface polymer engineering**. *Biomater Sci* 2020, **8**: 619–630, https://doi.org/10.1039/c9bm01232j.

32. He F, Liu H, Guo X, Yin BC, Ye BC: **Direct exosome quantification via bivalent-cholesterol-labeled DNA anchor for signal amplification**. *Anal Chem* 2017, **89**:12968–12975, https://doi.org/10.1021/acs.analchem.7b03919.

33. Choi ES, Song J, Kang YY, Mok H: **Mannose-modified serum exosomes for the elevated uptake to murine dendritic cells and lymphatic accumulation**. *Macromol Biosci* 2019, **19**:1–10, https://doi.org/10.1002/mabi.201900042.

34. Wang J, Bonacquisti EE, Brown AD, Nguyen J: **Boosting the biogenesis and secretion of mesenchymal stem cell-derived exosomes**. *Cells* 2020, https://doi.org/10.3390/cells9030660.

35. Gonzalez-King H, García NA, Ontoria-Oviedo I, Ciria M, Montero JA, Sepúlveda P: **Hypoxia inducible factor-1α potentiates jagged 1-mediated angiogenesis by mesenchymal stem cell-derived exosomes**. *Stem Cell* 2017, https://doi.org/10.1002/stem.2618.

36. Li Z, Mbah NE, Maltese WA: **Vacuole-inducing compounds that disrupt endolysosomal trafficking stimulate production of exosomes by glioblastoma cells**. *Mol Cell Biochem* 2018, **439**, https://doi.org/10.1007/s11010-017-3130-x.

37. Ludwig N, Yerneni SS, Menshikova Ev, Gillespie DG, Jackson EK, Whiteside TL: **Simultaneous inhibition of glycolysis and oxidative phosphorylation triggers a multi-fold increase in secretion of exosomes: possible role of 2′3′-cAMP**. *Sci Rep* 2020, https://doi.org/10.1038/s41598-020-63658-5.

38. Yang Z, Shi J, Xie J, Wang Y, Sun J, Liu T, *et al.*: **Large-scale generation of functional mRNA-encapsulating exosomes via cellular nanoporation**. *Nat Biomed Eng* 2020, https://doi.org/10.1038/s41551-019-0485-1.

39. Lespagnol A, Duflaut D, Beekman C, Blanc L, Fiucci G, Marine JC, *et al.*: **Exosome secretion, including the DNA damage-induced p53-dependent secretory pathway, is severely compromised in TSAP6/Steap3-null mice**. *Cell Death Differ* 2008, **15**:1723–1733, https://doi.org/10.1038/cdd.2008.104.

40. Rosenberger L, Ezquer M, Lillo-Vera F, Pedraza PL, Ortúzar MI, González PL, *et al.*: **Stem cell exosomes inhibit angiogenesis and tumor growth of oral squamous cell carcinoma**. *Sci Rep* 2019, https://doi.org/10.1038/s41598-018-36855-6.

41. Haraszti RA, Miller R, Stoppato M, Sere YY, Coles A, Didiot MC, *et al.*: **Exosomes produced from 3D cultures of MSCs by tangential flow filtration show higher yield and improved activity**. *Mol Ther* 2018, https://doi.org/10.1016/j.ymthe.2018.09.015.

42. * Patel DB, Luthers CR, Lerman MJ, Fisher JP, Jay SM: **Enhanced extracellular vesicle production and ethanol-mediated vascularization bioactivity via a 3D-printed scaffold-perfusion bioreactor system**. *Acta Biomater* 2019, **95**:236–244, https://doi.org/10.1016/j.actbio.2018.11.024.

This study uses 3D scaffold culture and ethanol to scale up EV production and improve vascularization activity. This is one of the first studies to conserve designed EV potency enhancement between 2D and 3D culture conditions on a large scale.

43. Zeh N, Schneider H, Mathias S, Raab N, Kleemann M, Schmidt-Hertel S, *et al.*: **Human CAP cells represent a novel source for functional, miRNA-loaded exosome production**. *PloS One* 2019, https://doi.org/10.1371/journal.pone.0221679.

44. Cheng Y, Zeng Q, Han Q, Xia W: **Effect of pH, temperature and freezing-thawing on quantity changes and cellular uptake of exosomes**. *Protein Cell* 2019, **10**:295–299, https://doi.org/10.1007/s13238-018-0529-4.

45. Charoenviriyakul C, Takahashi Y, Nishikawa M, Takakura Y: **Preservation of exosomes at room temperature using lyophilization**. *Int J Pharm* 2018, **553**:1–7, https://doi.org/10.1016/j.ijpharm.2018.10.032.

46. el Baradie KBY, Nouh M, O'Brien F, Liu Y, Fulzele S, Eroglu A, *et al.*: **Freeze-dried extracellular vesicles from adipose-derived stem cells prevent hypoxia-induced muscle cell injury**. *Front Cell Dev Biol* 2020, **8**:1–12, https://doi.org/10.3389/fcell.2020.00181.

47. * Bari E, Perteghella S, Catenacci L, Sorlini M, Croce S, Mantelli M, *et al.*: **Freeze-dried and GMP-compliant pharmaceuticals containing exosomes for acellular mesenchymal stromal cell

immunomodulant therapy. *Nanomedicine* 2019, **14**:753–765, https://doi.org/10.2217/nnm-2018-0240.

Using secretome characterization, the authors examine the effects on total protein, cytokine, and lipid content. This study demonstrates that EV isolation protocol may influence EV composition. This is the first study to define an acellular immunomodulatory dose that exhibits the same activity as MSCs.

48. Jeyaram A, Jay SM: **Preservation and storage stability of extracellular vesicles for therapeutic applications**. *AAPS J* 2018, **20**:1, https://doi.org/10.1208/s12248-017-0160-y.

49. Leiferman A, Shu J, Upadhyaya B, Cui J, Zempleni J: **Storage of extracellular vesicles in human milk, and MicroRNA profiles in human milk exosomes and infant formulas**. *J Pediatr Gastroenterol Nutr* 2019, **69**:235–238, https://doi.org/10.1097/mpg.0000000000002363.

50. Sancho-Albero M, Navascués N, Mendoza G, Sebastián V, Arruebo M, Martín-Duque P, *et al.*: **Exosome origin determines cell targeting and the transfer of therapeutic nanoparticles towards target cells**. *J Nanobiotechnol* 2019, **17**:16, https://doi.org/10.1186/s12951-018-0437-z.

51. Li B, Luan S, Chen J, Zhou Y, Wang T, Li Z, *et al.*: **The MSC-derived exosomal lncRNA H19 promotes wound healing in diabetic foot ulcers by upregulating PTEN via MicroRNA-152-3p**. *Mol Ther Nucleic Acids* 2020, **19**:814–826, https://doi.org/10.1016/j.omtn.2019.11.034.

52. Zhao B, Li X, Shi X, Shi X, Zhang W, Wu G, *et al.*: **Exosomal MicroRNAs derived from human amniotic epithelial cells accelerate wound healing by promoting the proliferation and migration of fibroblasts**. *Stem Cell Int* 2018, **2018**, https://doi.org/10.1155/2018/5420463.

53. Raposo G, Nijman HW, Stoorvogel W, Leijendekker R, Harding Cv, Melief CJM, *et al.*: **B lymphocytes secrete antigen-presenting vesicles**. *J Exp Med* 1996, **183**:1161–1172, https://doi.org/10.1084/jem.183.3.1161.

54. Phinney DG, Pittenger MF: **Concise review: MSC-derived exosomes for cell-free therapy**. *Stem cell* 2017, **35**:851–858, https://doi.org/10.1002/stem.2575.

55. Ferguson SW, Wang J, Lee CJ, Liu M, Neelamegham S, Canty JM, *et al.*: **The microRNA regulatory landscape of MSC-derived exosomes: a systems view**. *Sci Rep* 2018, **8**:1419, https://doi.org/10.1038/s41598-018-19581-x.

56. Khare D, Or R, Resnick I, Barkatz C, Almogi-Hazan O, Avni B: **Mesenchymal stromal cell-derived exosomes affect mRNA expression and function of B-lymphocytes**. *Front Immunol* 2018, **9**:3053, https://doi.org/10.3389/fimmu.2018.03053.

57. Wolfers J, Lozier A, Raposo G, Regnault A, Théry C, Masurier C, *et al.*: **Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming**. *Nat Med* 2001, **7**: 297–303, https://doi.org/10.1038/85438.

58. Lu J, Wu J, Xie F, Tian J, Tang X, Guo H, *et al.*: **CD4+ T cell-released extracellular vesicles potentiate the efficacy of the HBsAg vaccine by enhancing B cell responses**. *Adv Sci* 2019, **6**:1802219, https://doi.org/10.1002/advs.201802219.

59. * Fu W, Lei C, Liu S, Cui Y, Wang C, Qian K, *et al.*: **CAR exosomes derived from effector CAR-T cells have potent antitumour effects and low toxicity**. *Nat Commun* 2019, **10**:1–12, https://doi.org/10.1038/s41467-019-12321-3.

Using EV released from genetically engineered CAR-T cell, the authors demonstrate that CAR EVs do not express PD1. This study shows that CAR EVs are relative safe compared to CAR-T therapy and provide a promising cell-free therapeutic option. This is the first study to validate the antigenic specificity and cytotoxicity of CAR EVs.

60. Record M. *Exosomal lipids in cell-cell communication. Emerging concepts of tumor exosome-mediated cell-cell communication*, vol. 9781461436. New York: Springer; 2013:47–68, https://doi.org/10.1007/978-1-4614-3697-3_3.

61. Haraszti RA, Didiot MC, Sapp E, Leszyk J, Shaffer SA, Rockwell HE, *et al.*: **High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources**. *J Extracell Vesicles* 2016, **5**:32570, https://doi.org/10.3402/jev.v5.32570.

62. Haraszti R: **Engineered exosomes for delivery of therapeutic siRNAs to neurons**. *GSBS Dissert Theses* 2018, https://doi.org/10.13028/M2Z68X.

63. * Zhang K-L, Wang Y-J, Sun J, Zhou J, Xing C, Huang G, *et al.*: *Artificial chimeric exosomes for anti-phagocytosis and targeted cancer therapy*. 2019, https://doi.org/10.1039/c8sc03224f.

Using specific proteins and lipid from red blood cells and MCR-7 cancer cells, the authors demonstrate that lipid vehicles can achieve enhanced targeting and cellular uptake. This is one of the first studies to successfully create synthetic exosomes using multiple cell sources.

64. Jeschke MG, Rehou S, McCann MR, Shahrokhi S: **Allogeneic mesenchymal stem cells for treatment of severe burn injury**. *Stem Cell Res Ther* 2019, **10**:337, https://doi.org/10.1186/s13287-019-1465-9.

65. Tao H, Han Z, Han ZC, Li Z: **Proangiogenic features of mesenchymal stem cells and their therapeutic applications**. *Stem Cell Int* 2016, **2016**, https://doi.org/10.1155/2016/1314709.

66. Omoto M, Katikireddy KR, Rezazadeh A, Dohlman TH, Chauhan SK: **Mesenchymal stem cells home to inflamed ocular surface and suppress allosensitization in corneal transplantation**. *Invest Ophthalmol Vis Sci* 2014, **55**:6631–6638, https://doi.org/10.1167/iovs.14-15413.

67. Chen Z, Chen L, Zeng C, Wang WE: **Functionally improved mesenchymal stem cells to better treat myocardial infarction**. *Stem Cell Int* 2018, **2018**, https://doi.org/10.1155/2018/7045245.

68. Besse B, Charrier M, Lapierre V, Dansin E, Lantz O, Planchard D, *et al.*: **Dendritic cell-derived exosomes as maintenance immunotherapy after first line chemotherapy in NSCLC**. *OncoImmunology* 2016, **5**:1071008, https://doi.org/10.1080/2162402X.2015.1071008.

69. Kim OY, Dinh NTH, Park HT, Choi SJ, Hong K, Gho YS: **Bacterial protoplast-derived nanovesicles for tumor targeted delivery of chemotherapeutics**. *Biomaterials* 2017, **113**:68–79, https://doi.org/10.1016/j.biomaterials.2016.10.037.

70. Moreillon P, Majcherczyk PA: **Proinflammatory activity of cell-wall constituents from Gram-positive bacteria**. *Scand J Infect Dis* 2003, **35**:632–641, https://doi.org/10.1080/00365540310016259.

71. Kim OY, Choi SJ, Jang SC, Park KS, Kim SR, Choi JP, *et al.*: **Bacterial protoplast-derived nanovesicles as vaccine delivery system against bacterial infection**. *Nano Lett* 2015, **15**: 266–274, https://doi.org/10.1021/nl503508h.