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Review

Why Extracellular Vesicles Are Attractive Vehicles for RNA-Based Therapies?

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Abstract: Extracellular Vesicles (EVs) are a focus of intense research worldwide, with many groups exploring their potential for both diagnostic and therapeutic applications. Researchers have characterized EVs into various subtypes, modified common surface markers, and developed diverse isolation and purification techniques. Beyond their diagnostic potential, EVs are being engineered as delivery vehicles for various molecules and therapeutics. RNA therapeutics have the potential to be a transformative solution for patients suffering from chronic and genetic disorders and generally targeting undruggable targets. Despite the success of many RNA therapeutics in both in vivo studies and clinical trials, a significant challenge remains in effectively delivering these therapies to the target cells. Many research groups have adopted the use of lipid nanoparticles (LNPs) and other nanocarriers to encapsulate RNA therapeutics, aiming to deliver them as stably as possible to ensure optimal bioavailability and efficacy. While LNPs have proven successful as delivery vehicles, their use is not without drawbacks, such as accumulation within the body. EVs could be a potential solution to many of the problems around LNPs and other nanocarriers.

Keywords: extracellular vesicles; exosomes; modifications; hybrids; LNPs; engineering; cell-to-cell communication; signal transduction; diseases; therapy; RNA; cargo loading

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1. Introduction

Targeted drug delivery is gaining popularity over conventional drug delivery systems, such as tablets and syrups. Conventional drug delivery can suffer from poor bioavailability, uncontrolled drug release, the need for repeated drug administration, and fluctuations in the blood drug levels [1,2]. Drug delivery vehicles, such as nanoparticles, are currently favored because they address common issues associated with conventional drug delivery. These solutions include low toxicity, biocompatibility (not eliciting an immune response), and the ability to pass through biological barriers, such as the plasma membrane and even the blood–brain barrier. Additionally, targeted drug delivery methods reduce the need for repeated dosing and improve safety by using smaller amounts of drugs. These methods enhance drug bioavailability, chemical stability, and solubility [1,3,4]. Widely used delivery vehicles include viruses, lipid-based carriers such as liposomes and lipid nanoparticles (LNPs), and synthetic polymers. These vehicles deliver biologically active molecules, including proteins and nucleic acids such as mRNA, siRNA, shRNA, miRNA, and sgRNA-based ribonucleoprotein complexes [5–9]. Despite the mentioned delivery vehicles being more biocompatible than some conventional drugs, they can still elicit an immune response, prompting the development of more efficient and biocompatible delivery vehicles.

The knowledge of extracellular vesicles (EVs) has grown remarkably since their discovery around 40 years ago. Initially, EVs were thought to be merely a cell excretion pathway. However, researchers have since proven their importance in intercellular communication, as they transport materials such as proteins, lipids, nucleic acids, and other substances between cells [10,11]. EVs are now considered promising nanocarrier candidates for targeted drug delivery due to their physical properties, including small size, biocompatibility,

biostability, and safety. They can evade digestive enzymes and survive digestive fluids, making them suitable nanocarriers for the delivery of easily degradable drugs and other components. Additionally, their biostability and the ability to escape phagocytosis make them highly effective for long-distance biological communication. One of their unique characteristics is targeting specificity, which is highly dependent on the surface membrane composition that varies according to the cell of origin. EVs can be modified to carry specific molecules to enhance their recognizability of target cells. Once EVs reach the target cells (tissue), they can deliver their cargo through membrane fusion or phagocytosis [1,12,13].

Early clinical studies of EVs delivery to cancer patients did not cause any serious adverse effects. In these studies, autologous DC-derived EVs loaded with tumor antigenic peptides were administered to patients for the treatment of melanoma and non-small-cell lung cancer. While EV administration was found to be safe, the therapeutic benefits were limited [14]. The safety of EVs administration was further demonstrated by recent studies, where a single high-dose administration of human-derived EVs into mice did not trigger any acute inflammatory response [15].

Targeting macrophages is the next great frontier in cancer immunotherapy, and we are encouraged by the monotherapy anti-tumor activity exhibited by exoASO-STAT6 in preclinical models, which has not been observed among other approaches to date. Extracellular vesicles or exosomes facilitate the delivery of STAT6 antisense oligonucleotides, aiming to repolarize macrophages from the immunosuppressive M2 phenotype to the pro-inflammatory M1 phenotype, thereby potentially exerting antitumor effects [16].

In this review, we will discuss extracellular vesicles as targeted drug delivery vehicles and summarize various methods to isolate, purify, characterize, and modify EVs. Moreover, we will explore the use of RNA as a therapeutic molecule and highlight applications, key opportunities, and challenges in loading RNA into exosomes to create tissue-targeted therapy.

2. Extracellular Vesicles

'Extracellular vesicles' is a broad term for the heterogeneous populations of nanosized membranous particles released by eukaryotic and prokaryotic cells. They play a diverse role in intercellular communication and cargo delivery as they transport functional proteins, nucleic acids, and other metabolites to the recipient cells. EVs can be found in all types of mammalian fluids, such as serum, plasma, urine, breast milk, etc. [17–19].

Many researchers have proved that EVs influence several physiological processes, such as wound healing [20], protection against ischemia/reperfusion injury [21], modulating immune responses [22,23], cancer progression [24], and mediating cardiovascular diseases [25].

2.1. The Origin and Classification of Extracellular Vesicles

Extracellular vesicles are heterogeneous in size (ranging from 30 to 1000 nm), composition, cellular origin, and function [26]. EVs can be further classified by size depending on the cellular origin of the particle. Exosomes or small EVs are the smallest, 30–100 nm; they are derived from the endosomal pathway (Figure 1A), which is a process where the plasma membrane buds inwards, creating an endosome in the cytoplasm. Further inward budding of the endosomal membrane creates intraluminal vesicles (small vesicles inside the endosome), which leads to the formation of a multivesicular body (MVB). The MVB either fuses with lysosomes and is degraded or fuses with the plasma membrane, releasing the intraluminal vesicles into the extracellular space as exosomes [17,27,28].

Unlike exosomes, microvesicles, also called microparticles or ectosomes, are released by budding from the plasma membrane. Their size ranges from 100 nm to 1 μm [29]. They can also be referred to as large EVs [28].

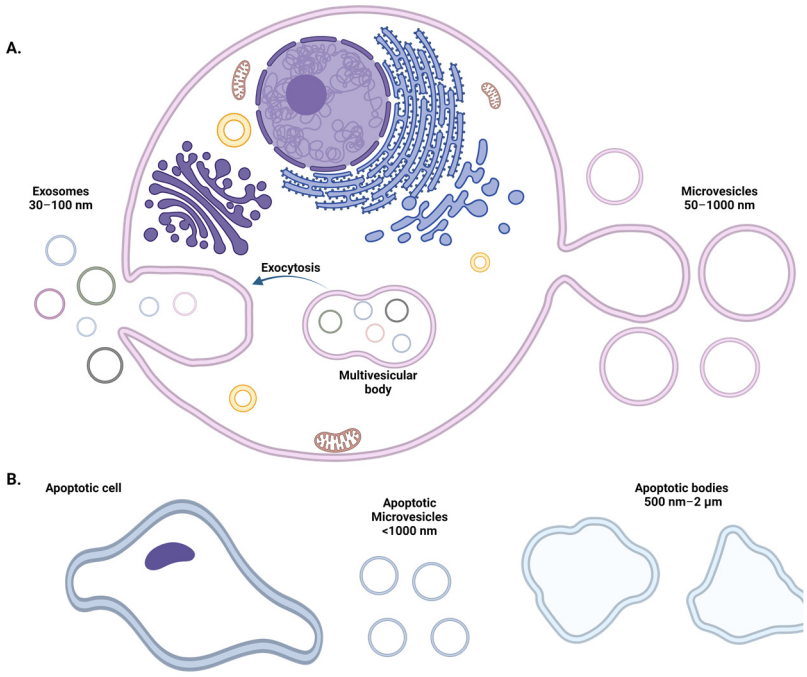

Figure 1. Biogenesis of extracellular vesicles and apoptotic bodies. (**A**). Exosomes are formed by fusing the multivesicular body with the plasma membrane and releasing them into the extracellular space. Microvesicles bud from the plasma membrane. (**B**). Dying cells release apoptotic bodies and apoptotic microvesicles.

Cells undergoing apoptosis (programmed cell death) are also known to produce different subsets of vesicles, termed apoptotic bodies and apoptotic vesicles, which often range from 500 nm to 2 µm in size and could also be as big as 5 µM [30] (Figure 1B). Like exosomes, the apoptotic bodies were thought to be garbage bags until it was discovered that they can deliver materials to healthy cells [31]. There is variability in the literature when it comes to the size range of the different subtypes of extracellular vesicles. Generally, exosomes are within the smallest size range, microvesicles are bigger than exosomes, and apoptotic bodies are the largest. Overlapping sizes among these three categories can also be found in the literature.

In the absence of accepted molecular markers to distinguish different EV subsets, they are currently mostly categorized based on their size differences. However, this might be challenged by the fact that small EVs (exosome-sized) can also bud directly from the plasma membrane. Osteikoetxea et al. [32] introduced protein-to-lipid ratio as a new parameter to categorize the subsets of extracellular vesicles. They found that the protein-to-lipid ratio increases with increasing diameter of the EV subpopulations. Additionally, they confirmed previous findings that exosomes are particularly rich in cholesterol and GM1 gangliosides [33]. They concluded that EV subpopulations can be categorized based on their membrane lipid order, which is a parameter that reflects the lipid packaging degree. The lipid order of membranes is a crucial parameter because it impacts signaling pathways. For instance, low-ordered membranes have lower protein–protein interactions, whereas membranes with high lipid order are typically found at the sites of cell adhesion, synapses, viral entry, and budding. The data suggest that exosomes are characterized by high membrane lipid order and high cholesterol content. These characteristics, among others, could explain the important role of exosomes in intercellular communication [32]. In this review, the terms extracellular vesicles and exosomes are used interchangeably. In theory, one can differentiate between EVs, an umbrella term, and exosomes, a subtype of EVs. However, in practice, it is not easy to isolate a pure particular subtype without the presence of other subtypes.

2.2. EVs' Composition

EVs are membrane-enclosed vesicles that can carry a myriad of molecules in their lumen and on their membranes. As the interest in EVs is growing, scientists are determined to further understand their composition, as they are potential biomarkers for diseases and are also important in several medical applications, such as targeted drug delivery [34]. The composition of extracellular vesicles is related to their cell of origin and, importantly, to the physiological state of the cell and is influenced by external stimuli acting on the cell at the time of EV release [35]. Different EV subsets carry different transmembrane and intraluminal molecules (Figure 2). The cargo can be composed of lipids, nucleic acids, proteins, and metabolites. Nucleic acids include dsDNA, ssDNA, and various RNA species, such as mRNA, miRNA, transfer RNA (tRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), mitochondrial RNA, mitochondrial DNA, and circular RNA [36]. Even though the composition of exosomes can be very heterogenous, some molecules are generally conserved across many populations and are considered EV markers. Among those molecules are the tetraspanins (CD9, CD63, and CD81), which are involved in several cellular functions, such as cell invasion, fusion, and penetration. Furthermore, small heat-shock proteins (HSP27, HSP60, HSP 70, and HSP90) are known to regulate the cellular responses and antigen presentation under stress and are also conserved in exosome populations [37]. TNFR is also found to be enriched in EVs of different origins [38]. MHCII molecules are only found on the EVs derived from immune cells [39]. EGFR enriched on EVs plays a crucial role in the tumor microenvironment [40]. Actin has been reported to be enriched in EVs [41]; so is tubulin [42]. Other classic EV markers are proteins involved in membrane fusion and transport, such as Annexin, Flotillin, GTPases, and Rab family members [43]. Endosomal markers, such as Alix and TSG101, are especially enriched in exosomes [44]. Lastly, EVs can also carry molecules such as integrins, proteoglycans, lectins, and ICAM-1, which play a role in the EVs' binding and uptake by cells [45].

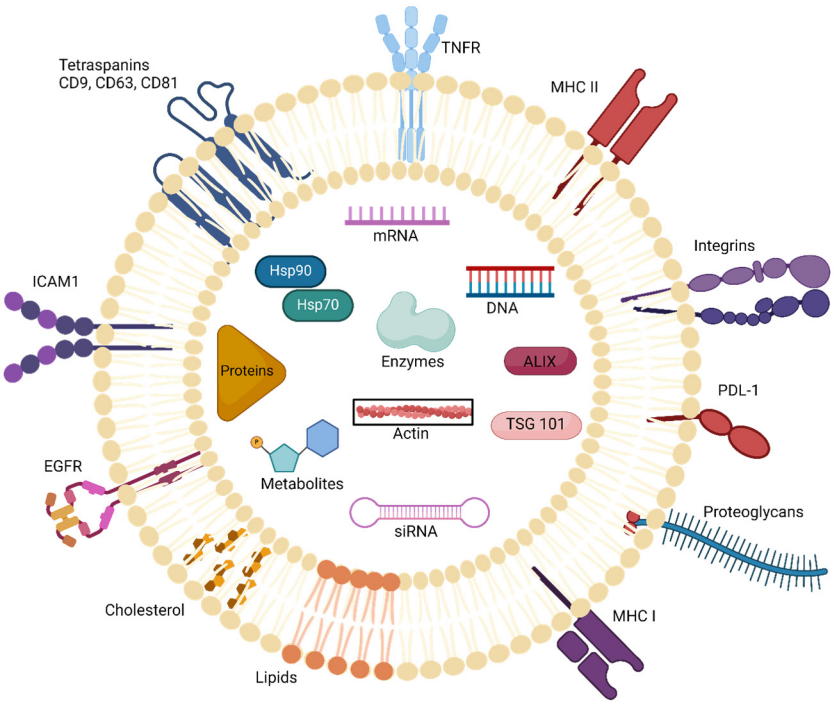

Figure 2. EVs' composition. EVs contain various transmembrane molecules, which include tetraspanins (e.g., CD63, CD9, and CD81), MHC molecules (I and II), integrins, ligands (e.g., PD-L1), receptors (e.g., EGFR, TNFR), and proteoglycans. Luminal substances include DNA, RNA, proteins (e.g., enzymes, ALIX), cytoskeletal proteins (e.g., actin, tubulin), and metabolites (e.g., sugars, fatty acids).

2.3. EVs' Isolation

A significant hurdle in this domain involves the isolation of extremely pure extracellular vesicles (EVs) or exosomes, ensuring a high recovery rate, and employing a rapid, cost-efficient approach. Here we discuss several popular EV isolation methods and bring about their pros and cons (Figure 3). The choice of the isolation and purification method can depend on several factors, including sample starting volume and application purposes. Additionally, some of the isolation techniques can be performed sequentially to obtain the best results in terms of purity and recovery.

Figure 3. Heatmap displaying the advantages and disadvantages of extracellular vesicle isolation methods based on purity, yield, concentration, throughput, scalability, cost of production, and required time.

Isolation by Ultracentrifugation: As simple as it sounds, this technique involves sedimentation of the particles by centrifugal force depending on their size and density. Accordingly, larger particles sediment first at lower centrifugal speeds, whereas smaller particles, i.e., exosomes, would sediment at higher speeds, i.e., 100,000× *g*. Ultracentrifugation (UC) is considered one of the most used methods to isolate EVs. It does not require much expertise or pre-sample preparations, and it is relatively affordable. However, it is labor-intensive and time-consuming. Differential UC (DUC) and gradient differential UC (GDUC) are also often used to isolate EVs. With DUC, the cell debris or any larger particles are first pelleted at 2000–2500× *g*; then the microvesicles/microparticles are pelleted at 10,000× *g*, and finally, exosomes or small EVs are pelleted at 10,000–150,000× *g*. Using GDUC, the EVs are also separated based on particle size and density; in this technique, however, the particles are placed in a density gradient medium. A sucrose gradient is usually used, where different concentrations of sucrose solution are layered on top of each other in a UC tube, and the sample is then layered on top (Figure 4A). Upon centrifugation at a high speed (100,000–200,000× *g*) for a long time (one to five hours), the particles will reside in the sucrose layer with a density equal to theirs [46,47]. Unlike differential UC, gradient differential UC offers higher separation efficacy and purity because EVs are not sedimented with residual proteins or other particles [48].

Immunocapturing: Also known as immunoaffinity isolation technique; the principle is based on the classic antigen–antibody interaction. Preferably, exosome membrane proteins, such as the tetraspanins CD9, CD63, and CD81, are used for capture. Magnetic beads, coated with monoclonal antibodies against the target protein (Figure 4B), are incubated with EVs, followed by exposure of the sample to magnetic force. The magnetic beads and the bound EVs are retained, and the rest of the sample is eluted and discarded. This method is highly specific and should result in very pure EVs [48,49].

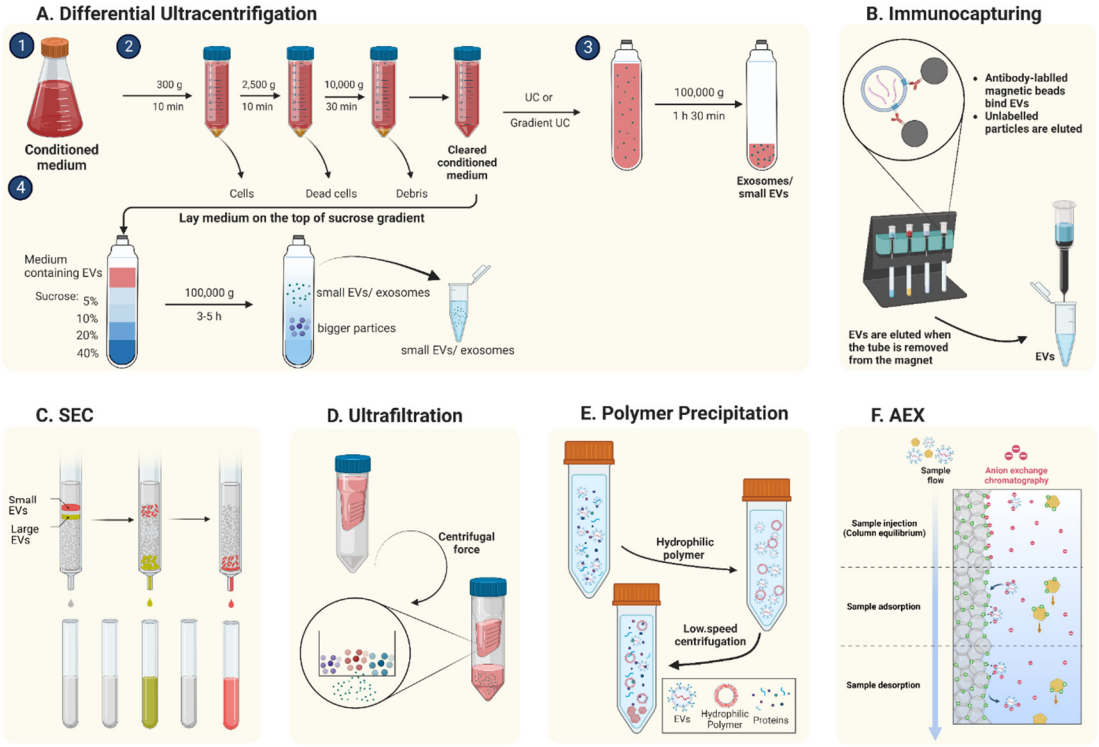

Figure 4. Extracellular vesicle isolation and purification methods (**A**). Differential centrifugation: (2) Live and dead cells as well as debris are sedimented out of the cell culture medium at increasing centrifugal speeds. (3) The cell culture medium then undergoes either UC at 100,000 g or is layered on top of a sucrose gradient and then centrifuged at 100,000 g (**B**). Immunocapturing: EVs are incubated with magnetic beads carrying antibodies against certain EV markers; the suspension is then added to a column placed on a magnet where the beads binding to EVs are captured and everything else is eluted; EVs are isolated when the column is removed from the magnet (**C**). Size Exclusion Chromatography (SEC): EVs and other small substances will enter the pores; larger particles will elute first, and smaller particles will be eluted in later fractions (**D**). Ultrafiltration: When centrifugal force is applied, the EVs pass through the filter, and the larger particles are retained (**E**). Polymer precipitation: non-water-soluble particles, such as EVs, will be expelled out of solution and sediment as a hydrophilic polymer binds water-soluble substances. The sediment can then be collected by centrifuging at low speed (**F**). Anion Exchange Chromatography (AEX): As the sample is applied to the column, the negatively charged EVs will bind the positively charged matrix while other substances are eluted. For sample collection, the EVs are desorbed from the matrix by changing the ionic strength of the buffer. Figure adapted from "Extracellular Vesicle Separation by Density Gradient Ultracentrifugation", "Chromatography 2 (Layout)", and "Anion and Cation Exchange Chromatography" using BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates. Accessed on 17 January 2023.

Size Exclusion Chromatography (SEC): SEC is a technique based on particle separation according to size. EVs are loaded in a column that contains a porous gel. Larger particles that cannot penetrate the pores will be eluted rapidly in the first fractions, and smaller particles will be retained and eluted at a slower rate in later fractions, as shown in Figure 4C. EVs isolated by SEC show high purity and high yield; the method also requires no preparation of the samples and does not require large sample volumes. However, this method requires equipment with high costs, and the procedure is time-consuming [50,51].

Ultrafiltration (UF): Like SEC, the main principle is the separation of particles based on the molecular size. The sample passes through a membrane with a specific pore diameter or molecular weight cut-off. Impurities or any larger particles that do not make the molecular weight cut-off will remain in the filter, and particles smaller than pore size will flow through.

A force is required to pull or push the sample through the filters. An example is the centrifugal force used along with tandem ultrafiltration, as shown in Figure 4D. Tangential Flow Filtration (TFF) could also be considered a UF technique, where pressure is used as the force to push excess fluid out of the filter to concentrate the sample. Ultrafiltration is easy and cost-efficient [41,42].

Polymer Precipitation: EVs are isolated by changing their solubility or their polydispersity. In this isolation method, polymers that bind water, such as polyethylene glycol (PEG), are incubated with the EVs. As the PEG molecules bind water, EVs and, most probably, other less soluble materials will be expelled out of the solution and precipitate. The sediment containing EVs can then be collected by ultracentrifugation (Figure 4E). This method is easy and scalable; however, the purity of the EV preparation is considered poor. There are many commercial kits available that employ this technique; ExoQuick (System Biosciences, Mountain View, CA, USA) is one of the most popular [47].

Anion Exchange Chromatography (AEX): In AEX, the negative charge on the surface of EVs [52] binds to the positively charged chromatography matrix. The EVs are then eluted from the column by washing with a buffer with higher ionic strength (Figure 4F). According to Heath et al., AEX is a quick and efficient way to isolate EVs. EVs isolated by AEX have better purity than EVs isolated by Tangential Flow Filtration, which means EVs purified by AEX do not have to undergo another purification step [53]. While purity is good, the yield and concentration are not the best when compared to other methods.

2.4. EVs' Characterization

Considering the difficulties coming along with the isolation of pure populations of EV subtypes, it is of utmost importance to fairly characterize the EV samples isolated before their analysis and/or application in the clinics. The importance of this matter is emphasized by the International Society of Extracellular Vesicles, which has set forth guidelines for characterizing EVs [43]. Table 1 shows the advantages and disadvantages of each characterization method discussed in this section.

Table 1. Summary of advantages and disadvantages of some of the common methods used to assess the quality of isolated extracellular vesicles and nanoparticles in general.

Method	Advantages	Disadvantages
Nanoparticle tracking analysis (NTA)	– Minimal sample preparation – Detects low particle concentration	– Difficulties in distinguishing EVs from other particles
Dynamic Light Scattering (DLS)	– Speed and simplicity – Minimal sample preparation	– Less accurate for heterogeneous samples
Tunable Resistive Pulse Sensing (TRPS)	– Measurement of EVs in biological samples without prior isolation – Reproducibility of data	– The measurable particle size range is determined by nanopore size. – Clogging of nanopores with large particles – Commercially available devices only from Izon Science, Ltd. (Christchurch, New Zealand)
Nano Flow Cytometry (NanoFCM)	– Quantitative – Measures at a single particle basis – Detects EV markers – Can distinguish artifacts and background noise from EVs	– Specific and high-maintenance equipment – Handling of equipment – Clogging issues
Bead-based Flow Cytometry	– Inexpensive – Feasible with any standard flow cytometer	– Semi-quantitative – Does not measure on a single particle basis

Table 1. *Cont.*

Method	Advantages	Disadvantages
Western Blot	– Sensitivity – Specificity	– Need for specific primary antibodies – Error-prone steps leading to inconsistent results – Need for protocol adaptations for each sample type – High amounts of starting material – Time- and labor-intensive – Semi-quantitative results
Transmission Electron Microscopy	– Allows for visualization of nanoparticles – Size and marker verification of isolated EV population	– Time- and labor-intense – Sample preparation protocol may influence things like size and morphology – Difficult quantification – Influence of disruptive factors

Nanoparticle Tracking Analysis (NTA) is acknowledged as one of the common techniques for quantifying EVs. This technique uses the ability of particles to scatter light in addition to the Brownian motion to determine the size and concentration of the particles in the suspension. When particles are suspended in liquids, the diffusion coefficient and hydrodynamic radius are calculated, accounting for the temperature of the system and fluid density [54].

Dynamic Light Scattering (DLS) is another common method used to characterize the size distribution and concentration of EVs. Like NTA, DLS employs Brownian motion, which measures size distribution based on the fact that smaller particles diffuse faster than larger particles. The particle size distribution is determined by the intensity of the light they scatter upon illumination by a laser beam. Additionally, DLS is also used to measure the zeta potential of the particles [55,56]. Zeta potential can be simply defined as the surface charge of EVs or other particles in solution [57]. The zeta potential reflects the colloidal stability and the tendency of aggregation of particles in the solution. Therefore, it is very important to investigate the zeta potential of EVs because it can influence biological processes, such as cellular uptake and cytotoxicity [58]. For instance, EVs with a zeta potential of +30 mV or higher are considered strongly cationic and could disturb the negatively charged plasma membrane [59].

Like DLS, Tunable Resistive Pulse Sensing (TRPS) can be used to measure the concentration, size distribution, and charge (Zeta potential) of nanoparticles in solution. This method was first reported in 2007 by Sowerby et al. [60]. In TRPS, resistive pulse sensing (RPS) is combined with a size-tunable nanopore in a membrane. An electric potential is applied to the stretchable membrane, which results in a stable and measurable ionic current. A combination of voltage and pressure is used to direct colloidal nanoparticles through the nanopore, and whenever a single particle crosses the pore, the current drops, resulting in a blockade signal or "resistive pulse". The analysis of this signal and its comparison to signals acquired with calibration particles with known properties allows the determination of the size/volume, which is equivalent to signal height, and charge, which is equivalent to the signal duration (the time it takes the particle to cross the pore) of the measured particle. In addition, the frequency of measured signals provides insight into the concentration of the measured particle. The first and so far, only, commercially available TRPS systems are provided by Izon Science, Ltd. [61].

NanoFlow cytometry (NanoFCM) is another important tool in the characterization of EVs. NanoFCM combines light scattering and fluorescence detection to measure the size distribution and concentration of EVs in addition to surface markers at a single-particle detection level [62,63]. Another way to assess the EVs is using beads-based flow cytometry. First, EVs are coupled to 4 µm beads, then the beads-coupled EVs are labeled with antibody(s) of interest, and the samples are washed and measured per normal protocol.

This method allows for easy semi-quantification of EVs and allows for the detection of several EV markers with any standard flow cytometer [64,65].

In Western Blot, samples are examined for the presence or absence of certain proteins of interest. First, protein lysates are separated by molecular weight via gel electrophoresis. Next, separated proteins are transferred to and immobilized on a membrane to allow for indirect target detection via antibody binding. However, this technique has several limitations, such as the need for the availability of primary antibodies that bind specifically to the protein of interest, inconsistent results originating from error-prone steps, and the need for protocol adaptations based on the sample type (reducing/non-reducing, denaturing/non-denaturing). In addition, quite high amounts of starting material are needed, the method is time-consuming and labor-intensive, and results are only semi-quantifiable [66,67].

Lastly, electron microscopy (EM) is commonly used for verifying the quality and integrity of extracellular vesicle preparations, allowing a morphologic analysis of the vesicles isolated. In transmission electron microscopy (TEM), samples must be dehydrated and fixed prior to analysis in a vacuum environment; hence, the morphology and size of the EVs is altered. This can be avoided by the analysis of samples under frozen conditions, known as cryo-TEM [68–70], making it possible to examine the naturally occurring structure of EVs [71]. However, EM requires long measurement times and only provides semi-quantitative results, which are operator- and protocol-dependent. Suboptimal and non-uniform vesicle adhesion can give a distorted impression about the particle size distribution. Hence, results must be compared to other techniques [61,62].

2.5. EVs' Engineering

Extracellular vesicles isolated from cell culture supernatant or different body fluids are often referred to as natural exosomes. However, natural exosomes have several drawbacks as a drug delivery vehicle in therapeutics. EVs' engineering or modification is a key process to improve their therapeutic potential and their use in clinics. In fact, EVs' engineering improves their tissue-targeting specificity, delivery efficiency by escaping the endosomal compartment, and potency while reducing their rapid clearance from the body and reducing the possibility of adverse immune reactions. EVs have been reported to be loaded with cargo such as miRNA, siRNA, nanoparticles, chemotherapeutic agents, and imaging probes. EVs can either be modified internally by modifying exosome cargo and/or through surface modifications, where the extravesicular membrane is modified.

2.5.1. Internal Modifications

Post Isolation Modifications: EVs have a lipid bilayer that surrounds a hydrophilic core. For any modification method to be successful, a complete understanding of the extracellular vesicles' structural characteristics, i.e., zeta-potential, surface proteins, and biology of the cell of origin, is crucial. Cargo or therapeutic materials can be incorporated into EVs post-isolation by two main approaches: active incorporation and passive incorporation, as illustrated in Figure 5A–E [1,72].

Passive Cargo Loading: Therapeutic materials or desired cargo can be incubated with the exosomes post isolation and purification. The drug is then expected to self-assemble into the lipid bilayer of exosomes (Figure 5A). This simple method has a minor trick: the drug must be hydrophobic to interact with the hydrophobic membrane of the exosomes. Curcumin is an example of an unstable, hydrophobic drug whose solubility, stability, and bioavailability increase when incorporated into EVs, namely exosomes; additionally, its anti-inflammatory activity also improves, according to Sun D. et al. [73].

Active Cargo Loading: Strategies for active loading of EVs post isolation and production include physically triggered strategies, such as electroporation, sonication, and freeze–thaw cycles (Figure 5B). These methods disrupt the membrane to allow for cargo entry inside the vesicles.

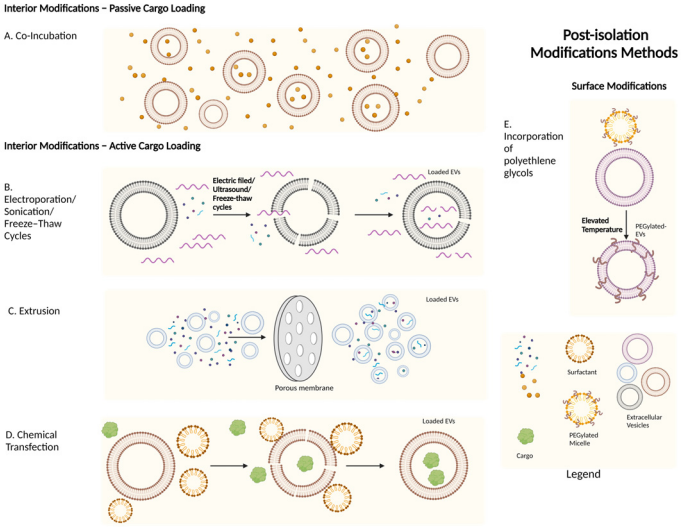

Figure 5. Extracellular vesicles post-isolation modification methods. Interior modifications: (**A**). EVs are co-incubated with cargo that passively diffuses into the EVs. (**B**). In electroporation, sonication, and freeze–thaw techniques, the membrane of the EVs is physically disrupted; cargo is then expected to diffuse into EVs through pores. (**C**). With the extrusion method, the membrane is disrupted by pushing the particles and the cargo through a porous membrane. (**D**). Chemical transfection method employs a surfactant to load the EVs. Surface Modification: (**E**). EVs can be incorporated with polyethylene glycol (PEG) by incubating them with PEGylated micelles.

Extrusion is one method that yields uniform-size EVs and is known for its high loading efficiency. As shown in Figure 5C, the technique includes pushing or extruding the EVs–cargo suspension through a porous membrane (pore size 100–400 nm). The cargo is then incorporated inside the EVs by agitating the membrane of the vesicles [1,47].

Electroporation involves incubating EVs with cargo, followed by the application of an electric field. This results in the formation of pores in the extracellular vesicles' membranes, and the cargo then defuses inside the EVs. Electroporation is one of the optimal methods to encapsulate siRNA. This method also has a major disadvantage as it can cause cargo aggregation, mainly for nucleic acids, due to electrochemical reactions, preventing their efficient loading inside the EVs.

Sonication is a technique where ultrasonic frequencies are used to disrupt the membrane of extracellular vesicles in suspension, which then allows cargo diffusion [46]. Sonication was effectively used for the transfer of nucleic acids into EVs. However, it often leads to the fragmentation of nucleic acids due to the shear forces applied during sonication. Nevertheless, for encapsulating certain high molecular weight compounds, such as catalase, into EVs [74,75], the sonication extrusion methods were described as the most efficient, followed by freeze and thaw cycles [75].

Another method to load cargo into EVs is the use of freeze–thaw cycles. The principle is similar to sonication, extrusion, and electroporation, where the membrane of EVs is disrupted to allow cargo loading (Figure 5B). In the case of freeze–thaw cycles, the vesicular membrane is disrupted by ice crystal formation and reforms during thawing [46]. A total of 5–10 freeze–thaw cycles at −80 °C and room temperature, respectively, are recommended [65]. This loading technique is mild and simple; however, the loading capacity is lower than sonication and electroporation [72].

In chemical transfection, EVs and cargo are incubated with a surfactant that increases the permeability of the membrane (Figure 5D), which allows for the penetration of cargo. Saponin is a frequently used surfactant to achieve loading. Thus, the method is often referred to as saponin-assisted loading or saponification [1,46]. Saponins were shown to be effective in loading hydrophobic molecules; however, they are known to have hemolytic activity, which could be quite harmful when used in in vivo studies. Therefore, it is

recommended to use minimal concentrations of saponin and to wash EVs thoroughly after cargo encapsulation [72,76].

2.5.2. Surface Modifications

Like interior modifications, surface modifications can also be achieved by acting on the parental cell using genetic engineering, simply using a co-incubation method, or by modifying the EVs post-isolation. Since the surface of the EVs is considered an important factor in biodistribution and affects the targeting potential of the drug, modifying the surface could be essential to achieve the desired targeting.

EVs' surface can be modified by using different components of synthetic lipid particles. As an example, PEGs (polyethylene glycols) have been incorporated into the EVs' membrane by incubation. This should increase the half-life and circulation time of EVs and prolong the exposure to their target-specific receptor [77]. Another approach is modifying proteins with a targeted sequence (PIP3, Prenylation domain) or by creating fusion proteins to tetraspanins [78]. To increase the cellular uptake of EVs and escape the endosomal pathway leading to improved functional delivery of the cargo, the membranes of EVs could be modified with arginine-rich cell-penetrating peptides [79].

2.6. EVs as Delivery Vehicles

Research is indeed going on in labs around the world to improve the quality of EVs as nanocarriers and to prove their function in battling different diseases, including cancer. For instance, Radha Munagala et al. [80] showed enhanced anti-cancer effects and increased drug bioavailability in vitro against A549 and H1299 (human lung cancer cell lines) when loading the chemo-preventative drug withaferin A (WFA) and chemotherapeutic drug paclitaxel (PAC) into exosomes, compared to the usage of free drugs. The drug-loaded exosome efficacy was also determined by the reduction of IC50 when compared to free drugs. They also proved that the exosome-loaded drugs suppress the lung tumor xenograft growth in vivo.

Another example is encapsulating Imperialine inside EVs to improve its delivery. Imperialine is a natural anti-inflammatory component from Bulbus *Fritillariae cirrhosae*; it has been shown to be effective against non-small-cell lung cancer (NSCLC) in pre-mature and early-stage tumors. Imperialine has a short half-life and an unfavorable biodistribution, which causes issues in clinical applications. To improve the delivery of the drug, EVs come to the rescue as favorable nanocarriers. In their study, Lin et al. [81] isolated EVs from human plasma, and Imperialine was loaded into the EVs using the micelle-aided method. They then attached CC8 ligands to the EVs to target the NSCLC. Their data show that loading the drug into EVs increased the anti-tumor activity by improving the targeting of the tumor and the drug retention.

Due to their many advantages, EVs are potential nanocarriers in many studies to treat and/or alleviate different classes of diseases and disorders. In fact, many of the studies made it through the pre-clinical phase and are currently in clinical trials (Table 2).

Table 2. Therapies involving exosomes that were approved for starting clinical trials. Examples from Clinicaltrials.gov.

Description	Exosome Source	Therapeutic Application	Sponsor/Company	Status	ClinicalTrials.gov Identifier	Ref.
iExosomes with KrasG12D siRNA to treat pancreatic cancer with KrasG12D mutations	Mesenchymal stromal cells	– Metastatic pancreatic adenocarcinoma – Pancreatic ductal adenocarcinoma – Stage IV pancreatic cancer AJCC v8	M.D. Anderson Cancer Center	Phase 1: Active, not recruiting	NCT03608631	[82]
CDK-004 (exoASO-STAT6) delivers STAT6 anti-sense oligonucleotide to M2 macrophages, resulting in anti-tumor activity.	WT and PTGFRN overexpressing HEK293 cells	– Advanced hepatocellular carcinoma (HCC) – Gastric cancer metastatic to liver – Colorectal cancer metastatic to liver	Codiak BioSciences	Terminated, Company bankruptcy	NCT05375604	[16]

Table 2. *Cont.*

Description	Exosome Source	Therapeutic Application	Sponsor/Company	Status	ClinicalTrials.gov Identifier	Ref.
CDK-003 (ExoIL-12) is composed of exosomes carrying a chain of the proinflammatory molecule IL-12 on its surface, which generates local and systemic anti-tumor activity.	Engineered HEK293 cells	– Cutaneous T-cell lymphoma (CTCL)	Codiak BioSciences	Phase 1 terminated, proceeding to Phase 2.	NCT05156229	[83]
CDK-002 (ExoSTING) are engineered exosomes that activate innate immunity locally in the tumor microenvironment.	WT, PTGFRN overexpressed, and PTGFRN knock out HEK293 cells	– Advanced solid tumor	Codiak BioSciences	Completed	NCT04592484	[84]
GDNPs are edible, non-toxic, and natural ginger-derived nanoparticles that exert anti-inflammatory properties and promote healing.	Ginger juice	– Irritable bowel syndrome	University of Louisville	Completed	NCT04879810	[85]

2.7. Native Cargo of Extracellular Vesicles

Extracellular Vesicles (EVs) naturally carry cargo derived from their cell of origin and reflect its state, which raises important questions regarding the fate of this native cargo when therapeutic molecules are loaded into the EVs. Specifically, one might wonder whether this native cargo could interfere with the functionality of the loaded therapeutic agents. Completely unloading or removing the native cargo from isolated EVs is a challenging and often impractical goal. However, certain procedures, such as electroporation or freeze–thaw cycles, can lead to the partial loss of native cargo from the population [86]. Just as the process of loading therapeutic cargo into EVs is challenging to fully control, the unloading of native cargo is similarly difficult to manage. As a result, some native cargo will inevitably remain within the EV population. The positive development is that this residual native cargo does not appear to pose a significant safety risk. Numerous biosafety studies conducted with both naïve and engineered EVs, in vitro and in vivo, have consistently shown that these EVs are generally safe [87,88].

3. Delivery Vehicles and Packaging Systems

3.1. Packaging Systems—Lipid Nanoparticles as a Gold Standard for Non-Viral RNA Delivery

In order to benefit from RNA molecules in curing diseases and/or alleviating symptoms, RNA must be delivered properly and protected from degradation by nucleases. Therefore, RNA molecules should be encapsulated or packaged to allow efficient delivery, cellular uptake, and intracellular release, hence improving the desired function of the RNA molecule.

One approach involves the use of lipid nanoparticles (LNPs), which are considered among the most advanced and promising non-viral RNA delivery systems for treating a broad range of diseases. LNPs' advantages and disadvantages are based on their components, i.e., structural lipids, cationic ions, stealth lipids, and cholesterol. For instance, using the appropriate ratio of cholesterol stabilizes the structure of the LNPs and interferes with the membrane permeability and elasticity [89,90]. The presence of cationic lipids is important for loading negatively charged nucleic acids. Although these cationic lipids can mediate efficient nucleic acid encapsulation and disrupt the endosomal membrane to enable cytoplasmic delivery, they also induce cytotoxicity and opsonization with plasma proteins, thus raising a safety red flag that indeed limits their use. Instead, pH-sensitive ionizable cationic amino lipids were developed. They are neutral in charge when administered and become ionized after cellular uptake [91]. One example is the ionizable amino lipid DLin-MC3-DMA (MC3), which is one of the LNP components used to deliver the first FDA-approved siRNA to treat hereditary transthyretin amyloidosis. Coelho et al. [92]

identified anti-transthyretin siRNA and encapsulated it in LNPs, generating ALN-TTR02, a drug that silences the transthyretin (TTR) gene, hence decreasing its production. In the phase I clinical trial, they noted an 82% TTR reduction [93]. ALN-TTR02 development was sponsored by Alnylam Pharmaceutics; they also completed two phase II clinical trials (ClinicalTrial.gov Identifier: NCT01617967 and NCT01961921) and are currently recruiting for a pregnancy surveillance program (ClinicalTrial.gov Identifier: NCT05040373). MC3 has its advantages but also has slow degradability in the body. This is a problem when it comes to the delivery vehicle of RNA therapeutics because, mostly, they require repeated dosing; thus, the vehicle must not accumulate [94]. One biodegradable option would be the L319-based LNPs, which are as potent and effective as MC3-based LNPs, but they have shown better elimination from the cellular compartments, according to Maier et al. [95].

3.2. Packaging Systems—Extracellular Vesicles

Nowadays, EVs have emerged as an attractive delivery vehicle for RNA drug delivery. What makes EVs very attractive delivery vehicles is their low toxicity, hypo immunogenicity, and biocompatibility. Other advantages of EVs as a packaging system also include their ability to avoid drug-degrading enzymes, hence extending the half-life of RNA drugs during delivery. Correspondingly, due to their phospholipid bilayer membrane, the bioavailability of the drug after loading is improved. Another advantage is their small size, even on the nanoscale, which allows them to diffuse among cells [1,11,12]. These advantages make them highly attractive as packaging systems, especially after some studies showed that they are tolerated after repeated dosing. Mendt et al. showed that MSC-derived exosomes did not trigger an adverse immune response in vivo despite the repeated dosing (every 48 h for 4 months) [96]. As EVs are a natural packaging system for different RNA molecules, it is only sensible to load RNA into EVs to target certain cells. In practice, however, the use of RNA-loaded EVs in clinical development is hampered by their low loading efficiency.

3.3. Hybrid Particles

As already discussed, both LNPs and EVs have their pros and cons (Figure 6). An alternative approach is forming a hybrid that combines the anticipated benefits of biological nanoparticles, namely EVs, such as biocompatibility and better delivery efficiency, and the properties of synthetic particles, i.e., LNPs, including high loading efficiency. A common method for loading employs nanoparticles loaded with a compound of interest, which are then co-incubated with EVs, allowing their fusion by different techniques [97]. Sato et al. [98] used freeze–thaw cycles for the fusion of EVs with loaded liposomes containing a compound of interest. Chemical transfection approaches are also available to load cargoes in the EVs, using, for instance, lipofectamine, but this method is not applicable for in vivo studies due to the toxicity of the cationic lipid. This approach also has the inability to separate EVs and liposome micelles. Nonetheless, the use of lipid-based vehicles to efficiently deliver cargo to EVs, forming hybrid EVs or hybridosomes, has been in fact attracting great attention over the most recent years, triggering the issuing of patents [99] and further development and commercialization of the technology within novel EV-based companies, such as Anjarium. Evers M. and P. Vader et al. published their EV–liposome hybrid nanoparticles as a siRNA delivery vehicle. They noted that the hybrid has increased biocompatibility and lower in vitro toxicity in comparison to the liposomes. However, these data were limited to a certain cell type, suggesting cell-specific interactions. They also showed that hybrids still retain the functional properties of EVs, i.e., the cell of origin [100].

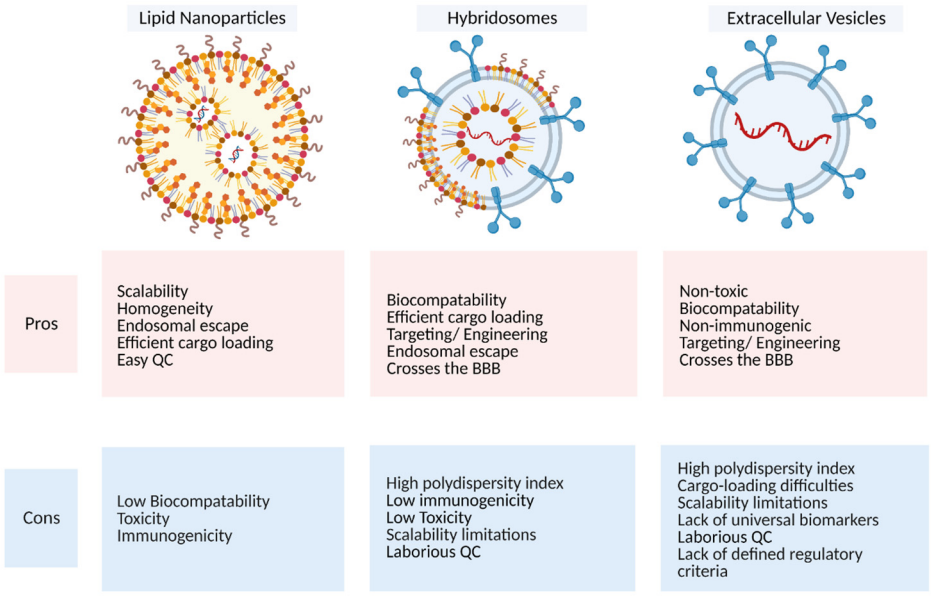

Figure 6. Pros and cons of extracellular vesicles, lipid nanoparticles, and hybridosomes as targeted drug delivery vehicles.

4. RNA as a Therapeutic Molecule

RNA-based therapeutics have great potential to target a large part of the currently undruggable genes, but there are significant hurdles around deliverability and stability, explaining the need for appropriate delivery systems. Generally, RNA delivery hurdles include efficiency of delivery and uptake, tissue bioavailability, circulatory half-life, and repeated dosing. Repeated dosing in chronic diseases is considered one of the major challenges because even if the RNA molecule and its carrier, such as lipid nanoparticles (LNPs), are improved and optimized to have lower immunogenicity, repeated dosing might eventually elicit an immune response [91,101].

mRNA vaccines have conquered the vaccination world and possibly changed it forever. Additionally, these vaccines, which have been released into the market, have opened a wide door of possibilities in the world of RNA therapeutics. However, unlike the mRNA vaccines, using RNAs as therapeutics will not be as smooth. To elicit an immune response, a minimal amount of protein, and hence, mRNA, is required. However, 1000-fold more of the translated protein is required to reach a therapeutic threshold [91].

One marvelous naturally occurring tool is RNA interference (RNAi). RNAi inhibits the translation of pathological proteins in a sequence-specific way. This process is executed by short interfering RNA (siRNA), which serves as a guide for the RNA-induced silencing complex (RISC), which in turn binds the target sequence and cleaves the RNA, thus preventing its translation [102]. Indeed, RNAi is an attractive technique that opens doors to possibilities for treating many diseases and disorders; however, siRNAs cannot be delivered without a vehicle because (a) they cannot reach the cytosol of target cells on their own, and (b) they are unstable and immunogenic [100].

4.1. RNA Cargo Loading into EVs

Therapeutic RNA can be loaded into EVs prior to EV isolation, based on the engineering of the secreting cells. The technique relies on the physiological pathways of intracellular trafficking of RNA into EVs during their biogenesis. One example of RNA incorporation into EVs through its pre-loading in parental cells was described by Yang et al., where miR-124 was overexpressed in MSC-originating EVs loaded with miR-124. These EVs are currently being tested for the treatment of acute ischemic stroke in a clinical trial (ClinicalTrial.gov Identifier: NCT03384433) [103]. Physiological RNA loading into EVs is described to be dependent mainly on the presence of specific sorting sequences in the target

RNA cargo and their binding by RNA-binding proteins, such as hnRNPA2B1 [104] and SYNCRIP [105]. This concept was particularly explored in the work of Hung et al., which described a platform for targeted and modular EVs loading with engineered RNA, the TAMEL platform [106]. To date, several RNA sequences have been identified as promoting RNA loading into EVs. For instance, Bolukbasi et al. described a consensus sequence present in the 3′UTRs of mRNA enriched in tumor cell MVs that promoted two-fold mRNA enrichment in EVs. Thus, identifying zip-code-like signal sequences can be used to target mRNA into MVs [107]. Other short-sequence motifs, such as EXOmotifs and Clmotifs, were also identified as determining the fate of miRNA [108]. These methods are specifically used to load cargo into the EVs. Another example of RNA loading into EVs explores different pathways of EV biogenesis. ARRDC1-mediated microvesicles, a subtype of EVs originating from the cell surface, were recently reported to be loaded with the CRISPR/Cas9 tools. The genome-editing CRISPR-Cas9/guide RNA complex was delivered via arrestin domain containing protein 1 [ARRDC1]-mediated microvesicles (ARMMs) to the targeted cells by fusing Cas9 with the WW domains, which interact with the PPxY motifs of ARRDC1 [9]. The authors demonstrated the functional delivery of the tumor suppressor p53 protein in vivo using this method. Another approach to loading RNA into EVs is based on their modification post-isolation. Recent studies from the R. Kalluri group demonstrated electroporation as an efficient loading approach for siRNA cargo targeting oncogenic mutant KRAS into EVs derived from BJ fibroblast. This resulted in EVs successfully suppressing the cancer growth of pancreatic ductal adenocarcinoma in vivo and better mice survival [8]. One more recent study from Usman et al. updated that RBCEVs can be used as versatile therapeutic delivery vehicles for many cargoes, like ASO that antagonizes miR-125b, small gRNAs, as well as long RNAs such as cas9 mRNA targeting any gene of interest, posing no risk for horizontal gene transfer as they are enucleated [109].

4.2. The Delivery of RNA-Loaded EVs into Target Cells

For the RNA molecules to function as intended, they must first enter the target cell and reach the cytoplasm. The RNA molecules will be enclosed by the EVs; hence, the EVs could unpack their cargo at the cell membrane by direct fusion, or it could enter the cell through endocytosis, phagocytosis, or macropinocytosis. EVs or any other carrier nanoparticles should have the keys to enter the target cells; these keys are certain receptors that can be engineered on the surface of the EVs to bind with the receptors/ligands on the target cell surface. One example is incorporating fusion proteins, syncytin1 and syncytin2, onto the surface of the EVs, which can bind to receptors on target cells and facilitate the fusion [110].

Even if the delivery to the cells is successful, the actual delivery and functionality of the RNA molecules are not guaranteed, as each entry pathway poses its own challenges, such as degradation by the lysosomes in the cytoplasm [106,111] One solution is the endosomal escape, which will be described in the next pages.

Researchers also wanted to track the EV delivery pathways inside the cell. However, due to their small size, highly sensitive methods and specific markers are required for the molecular tracking of EVs. Toribio et. al. developed [112] a quantitative method to track EVs after delivery to the cells (EV uptake) based on two reporter proteins, DSP1 and DSP2. As split molecules DSP1 and DSP2 reunite, they produce the full form of a green fluorescent and luminescent protein. They fused DSP1 to either the N-terminus of CD9 or CD63 (tetraspanins), and eukaryotic cells were transfected with DSP2. When the DSP1-expressing EVs are successfully delivered into the cells expressing DSP2, the DSP1 protein will reunite with the DSP2, which activates the two reporter signals.

When the RNA molecules have been delivered to the cytosol and managed to avoid RNA degradation, they might be able to create a functional response.

4.3. Applications of RNA-Loaded EVs

Dong et al. [87] used microfluidic electroporation in addition to step pulse stimulation to load IFN-g-mRNA into small EVs (another term for exosomes). They also engineered

EVs to overexpress CD63, which will be the key to entering the glioblastoma cells. After treatment, the mRNA-EVs bind to the anti-PDL1 and/or anti-CD71 antibodies, which are overexpressed by the glioblastoma tumor. The EVs then enter the cells mainly by clathrin-mediated endocytosis. After entry, the mRNA is translated to produce IFN-g, which upregulates the expression of MHC-I on the glioblastoma cells, hence affecting their immunogenicity, as the glioblastoma and other solid tumors tend to downregulate MCH-I to evade immune surveillance by avoiding antigen presentation [113]. After confirming the results in vitro using glioblastoma cell lines, they evaluated the biosafety of the EVs. There was no observed hemolytic toxicity. Additionally, the biosafety and biocompatibility study in vivo showed no deviation from the normal range of serum markers. The engineered EVs also did not alter the total red blood cell and white blood cell counts. In vivo studies in a murine glioblastoma model showed that the mice treated with IFN-g-mRNA EVs every 3 days showed an inhibition of tumor growth and an extension of survival time: media of 53 days for the treated mice and 29 days for control group mice. After treatment, the INF-g protein expression was elevated, which resulted in the upregulation of the MHC-1 receptors. The MCH-1 upregulation is correlated with the increase in the CD8+ T-cells in the tumor microenvironment (TME). The increase in INF-g is also correlated with the increase in M1-macrophages in the TME. These EVs managed to reprogram the TME, which allowed the immune system to regain control and act by increasing the infiltration of immune cells.

A study by Dong et al. [87] investigated many of the topics discussed in this review, such as the ability of EVs to cross the blood–brain barrier to reach the tumor, the efficacy of the mRNA therapeutics, and the efficacy of EVs as a packaging system and a delivery vehicle due to their ability to be engineered and manipulated. They also tested for toxicity on different levels, including toxicity to organs such as the heart, liver, lungs, spleen, and kidneys. They also showed that the EVs as a packaging system do not induce a systematic or local immune response, which adds to their score in biocompatibility.

In 2016, Didiot et. al. [114] explored exosomes as packaging and delivery solutions to overcome the obstacle of delivering oligonucleotides to treat Huntington's disease. They used the co-incubation method to load hydrophobic modified siRNA^{HTT} (modified) into exosomes. The siRNA^{HTT} exosomes did not induce cytotoxicity or activate the immune system. In vivo, the siRNA^{HTT} exosomes were internalized by primary cortical neurons and showed a dose-dependent silencing of Huntington's mRNA (mRNA^{HTT}). In a previous study performed by Kordasiiewicz et al. [115], they used about 140 to 700 µg of anti-sense oligonucleotides (ASO) over two weeks to silence the mRNA^{HTT}. The mRNA^{HTT} was reduced by about 38% at the 2-week time point, and the reduction persisted for 12 weeks; then, the mRNA^{HTT} levels rose again after 16 weeks. They used transient infusion to deliver the ASO to the CSF. While using exosomes as a delivery vehicle, Didiot et. al. [114] managed to silence about 35% of the Huntington's mRNA in the brain using 3.5–7 µg/mL of siRNA^{HTT} over the course of 1 week (1 µg/day). As mentioned earlier, the use of nanocarriers should allow for using less concentrations of the drug and reduce repeated dosing.

One application of the targeted delivery of RNA using EVs is currently in phase 1 clinical trials: iExosomes with KrasG12D siRNA to treat pancreatic cancer with KrasG12D mutations (ClinicalTrial.gov Identifier: NCT03608631) [82]. The KRAS G1D mutation is the cause of the majority of pancreatic cancer cases. Therefore, silencing the KRAS G12D gene is a potential therapeutic approach [116].

5. Limitations in the Filed

As discussed, the RNA molecules are negatively charged and cannot penetrate the anionic phospholipid bilayer of the cell membrane. Therefore, benefiting from RNA therapeutics is dependent on chemical modifications and protection technologies to ensure stability and delivery. Otherwise, most of the administered RNA dose will be degraded by nucleases or simply engulfed by the cells of the innate immune system. An important

function of the delivery vehicle is to aid the RNA molecules in escaping the endosome degradation inside the cell. Otherwise, only a tiny fraction of the administered RNA will function in the cell and the rest will be degraded. This will indeed result in the need for repeated dosing or increasing the dose to reach and maintain the therapeutic dose [117]. Additionally, the size and concentration of the nanoparticle that carries therapeutic molecule matter because it impacts the stimulation of the innate immune system, as well as the efficacy of its cellular and tissue distribution [118].

5.1. Endosomal Escape

Aside from all the positives of using extracellular vesicles for therapeutic applications, exosomes are taken up by cells through endocytosis, and once inside the endosomes, the cargo can be degraded before reaching its target site [119]. This will indeed result in the need for repeated dosing or increasing the dose to reach and maintain the therapeutic dose. Therefore, various strategies have been developed to modify exosomes to enhance their endosomal escape and improve cargo delivery. An interesting approach is to modify the surface of the exosomes with pH-sensitive peptides or proteins that can fuse the exosome membrane and the endosomal membrane [120]. For example, researchers have shown that fusing a peptide derived from the influenza virus hemagglutinin protein to the membrane of the EV can enhance the release of cargo from endosomes. Similarly, attaching a pH-sensitive fusogenic peptide derived from the Ebola virus glycoprotein to EV has been shown to promote endosomal escape and enhance the delivery of small interfering RNAs (siRNAs) to the target cells, [111,121]. Another approach is to engineer exosomes to express membrane proteins that can mediate endosomal escape. For example, researchers have genetically engineered exosomes to express the lysosomal-associated membrane protein 2B (LAMP2B) on the surface of exosomes, which has been shown to promote endosomal escape and enhance the delivery of therapeutic proteins to the target cells [4]. Furthermore, the use of natural or synthetic compounds to enhance exosome-mediated cargo delivery has been extensively explored. One notable example is the employment of ionizable lipids, which can facilitate the escape of nanoparticles from the endosome, thereby significantly improving cargo delivery. Ionizable lipids possess the unique ability to undergo protonation at the acidic pH found within endosomes, leading to the destabilization of the endosomal membrane and the subsequent release of the exosome cargo into the cytoplasm. This approach not only enhances the efficiency of exosome-based delivery systems but also addresses a critical intracellular barrier to effective RNA delivery, thereby augmenting their therapeutic potential [122]. These advancements underscore the promise of integrating exosome technology with innovative molecular strategies to optimize the delivery and therapeutic impact of RNA therapeutics in clinical settings.

Overall, modifying exosomes to enhance their endosomal escape is an active area of research, and several promising strategies have been developed to improve the delivery of therapeutic cargo to target cells.

5.2. Targeted Delivery to Specific Cell Type

The precise delivery of drugs to specific sites within the body for disease treatment remains a significant challenge in the field of RNA therapeutics. Decades ago, the introduction of nanoparticles as more efficient drug delivery systems aimed to mitigate adverse side effects in patients [3]. To address this challenge, EVs can be surface functionalized with targeting moieties capable of recognizing specific targets, predominantly present at the delivery site [103,123]. These targeting molecules can be categorized into four primary groups: antibodies, oligonucleotide aptamers, targeting peptides, and miscellaneous molecules [124]. Antibodies, while highly specific, are costly and pose immunogenicity concerns. Oligonucleotide aptamers and peptides, with lower affinities but cost-effective production and easier functional modifications, have garnered increasing attention, boasting better stability and protease resistance compared to antibodies [4]. Peptides, being smaller and more amenable to in silico selection, offer advantages in terms of speed and

reliability [125]. Peptides, developed experimentally or via in silico methods, have emerged as promising targeting molecules. Computational modeling aids in expediting experimental procedures and enhancing peptide features, including selection, binding affinity, stability, and specificity, thereby expanding the repertoire of available sequences and their modifications for various targets [126,127].

Extracellular vesicles' surface modifications to enhance targeting could be achieved by genetic engineering and chemical modifications. Genetic engineering involves merging the gene sequence of a protein or a peptide typically displayed on target cells with that of a specific exosome membrane protein. One example is LAMP-2B, which is commonly used for surface display. LAMP-2B, part of the lysosome-associated membrane protein family, is primarily located in lysosomes and endosomes but is also present on the cell surface. It can be engineered to display targeting sequences on the exosome surfaces. LAMP-2B is highly expressed on dendritic cell-derived exosomes, and its N-terminus, exposed on the exosome surface, can be modified with targeting sequences. Genetic engineering is effective for displaying peptides and proteins on the exosome surface, but it is limited to motifs that can be encoded genetically. On the other hand, chemical modification allows for a wider range of natural and synthetic ligands to be attached to exosomes through conjugation reactions or lipid assembly. Conjugation reactions can modify exosome surface proteins in a stable, covalent manner, but the complex surface structure of exosomes can reduce the efficiency of these reactions, and they often lack precise site control. Additionally, covalent modifications may alter the exosome's structure and function [128,129]. Peptides that specifically bind to certain organs or tissues can be identified using phage-display technology [130] and then genetically attached to the N-terminus of LAMP-2B for targeted delivery. Donor cells transfected with plasmids encoding these modified peptides generate exosomes displaying the engineered ligands on their surface. For instance, the rabies virus glycoprotein (RVG) peptide (TIWMPENPRPGTPCDIFTNSRGKRASNG), which specifically binds to acetylcholine receptors, has been utilized to create neuro-targeted exosomes for drug delivery to the central nervous system. In one example, RVG-modified exosomes loaded with miRNA-124, when injected intravenously, were able to reach ischemic areas in the cortex and stimulate neurogenesis [103]. iRGD-modified exosomes have been used to deliver the KRAS siRNA specifically to the $\alpha v\beta 3$-expressing A549 tumors in vivo, leading to targeted KRAS gene knockdown and the suppression of tumor growth [131]. Additionally, the tLyP-1 peptide (CGNKRTR), which selectively targets neuropilin-1 (NRP1) and neuropilin-2 (NRP2) receptors, has been used to deliver siRNA specifically to human non-small-cell lung cancer (NSCLC) cells [132].

While less explored, chemical modification presents a promising approach for customizing exosome surfaces for targeted drug delivery. For example, the amine groups on the exosome proteins can be readily modified with alkyne groups, enabling their attachment to azide-containing reagents via copper-catalyzed azide-alkyne cycloaddition (CuAAC) "click" reactions [133]. Jia G. et. al. [134] have effectively applied click chemistry to modify exosome surfaces by linking them to the glioma-targeting RGE peptide (RGERPPR). Moreover, these exosomes chemically linked to the glioma-targeting RGE peptide (RGERPPR) have demonstrated the capacity to cross the blood–brain barrier (BBB) efficiently and selectively target tumor sites, showing significant anticancer effects in tumor-bearing mice. Similarly, EVs linked to the peptide c (RGDyK) through biorthogonal click reactions have effectively crossed the blood–brain barrier (BBB) and reduced inflammation and cell apoptosis in a mouse model of transient middle cerebral artery occlusion [135]. Chemical modifications have also enabled the conjugation of large biomolecules onto exosomes, such as the design of an exosome-based immune checkpoint blocker that interferes with the CD47-SIRPα checkpoint on tumor cells, enhancing immune cell-mediated tumor cell engulfment, as Koh et. al. showed in their research [136,137]. Inserting amphipathic molecules into the exosome lipid bilayer is another promising approach. Exosomes modified with the DSPE-PEG-based ligands, such as DSPE-PEG-RGD, have shown potential for targeted drug delivery to tumors. When combined with folate, these exosomes accu-

mulate in tumor sites, improving uptake and therapeutic effectiveness in vivo [138,139]. Furthermore, chol-conjugated exosomes have been explored for targeted delivery of nucleic acids and therapeutic agents to tumor tissues, offering enhanced stability and promising outcomes in antitumor efficacy [140]. These findings underscore the potential of chemical modification strategies to enhance the targeting and therapeutic efficacy of exosome-based drug delivery systems [141]. Each of these strategies presents unique challenges, highlighting the need for further research to optimize exosome surface engineering for targeted therapeutic delivery [17,142–146].

6. Conclusions

In recent decades, there has been a growing interest in exploring the exosomes' potential as drug delivery systems, particularly in cancer treatment, where combination therapy involving drugs and nucleic acids delivered via exosomes is gaining traction. Clinical trial registrations on clinicaltrials.gov reveal a notable number of ongoing trials investigating the exosomes' potential, including those focusing on loading exosomes with RNA molecules. Exosomes offer distinct advantages as carriers for RNA therapeutics. Firstly, they are naturally occurring and can be sourced from various cell types, offering a broad range of targeting options and potentially reducing immunogenicity. Secondly, exosomes efficiently transport diverse molecules, including RNA and small molecules, in a protected manner, enhancing their stability and half-life. Lastly, exosomes possess tissue-targeting mechanisms, enabling them to deliver drugs directly to the affected tissues and organs. While various strategies have been employed to load short nucleic acids, such as siRNA, shRNA, and mRNA, into exosomes, efficiently encapsulating large mRNAs remains a challenge. To advance exosome-based RNA therapeutics, achieving cost-effective RNA cargo loading into the exosomes coupled with surface modifications for targeted delivery is imperative. Therefore, further attention should be directed towards establishing large-scale RNA-loaded exosomes for clinical applications.

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References

1. Kučuk, N.; Primožič, M.; Knez, Ž.; Leitgeb, M. Exosomes Engineering and Their Roles as Therapy Delivery Tools, Therapeutic Targets, and Biomarkers. *Int. J. Mol. Sci.* **2021**, *22*, 9543. [CrossRef] [PubMed]
2. Adepu, S.; Ramakrishna, S. Controlled Drug Delivery Systems: Current Status and Future Directions. *Molecules* **2021**, *26*, 5905. [CrossRef] [PubMed]
3. Wilczewska, A.Z.; Niemirowicz, K.; Markiewicz, K.H.; Car, H. Nanoparticles as drug delivery systems. *Pharmacol. Rep.* **2012**, *64*, 1020–1037. [CrossRef] [PubMed]
4. Liang, Y.; Duan, L.; Lu, J.; Xia, J. Engineering exosomes for targeted drug delivery. *Theranostics* **2021**, *11*, 3183–3195. [CrossRef] [PubMed]
5. Valadi, H.; Ekström, K.; Bossios, A.; Sjöstrand, M.; Lee, J.J.; Lötvall, J.O. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* **2007**, *9*, 654–659. [CrossRef]
6. Thakur, B.K.; Zhang, H.; Becker, A.; Matei, I.; Huang, Y.; Costa-Silva, B.; Zheng, Y.; Hoshino, A.; Brazier, H.; Xiang, J.; et al. Double-stranded DNA in exosomes: A novel biomarker in cancer detection. *Cell Res.* **2014**, *24*, 766–769. [CrossRef]
7. Kalra, H.; Drummen, G.P.C.; Mathivanan, S. Focus on Extracellular Vesicles: Introducing the Next Small Big Thing. *Int. J. Mol. Sci.* **2016**, *17*, 170. [CrossRef]
8. Kamerkar, S.; LeBleu, V.S.; Sugimoto, H.; Yang, S.; Ruivo, C.F.; Melo, S.A.; Lee, J.J.; Kalluri, R. Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature* **2017**, *546*, 498–503. [CrossRef]
9. Wang, Q.; Yu, J.; Kadungure, T.; Beyene, J.; Zhang, H.; Lu, Q. ARMMs as a versatile platform for intracellular delivery of macromolecules. *Nat. Commun.* **2018**, *9*, 960. [CrossRef]

10. Harding, C.V.; Heuser, J.E.; Stahl, P.D. Exosomes: Looking back three decades and into the future. *J. Cell Biol.* **2013**, *200*, 367–371. [CrossRef]
11. Wang, Y.; Zhang, Y.; Cai, G.; Li, Q. Exosomes as actively targeted nanocarriers for cancer therapy. *Int. J. Nanomed.* **2020**, *15*, 4257–4273. [CrossRef] [PubMed]
12. Amiri, A.; Bagherifar, R.; Ansari Dezfouli, E.; Kiaie, S.H.; Jafari, R.; Ramezani, R. Exosomes as bio-inspired nanocarriers for RNA delivery: Preparation and applications. *J. Transl. Med.* **2022**, *20*, 125. [CrossRef] [PubMed]
13. Akuma, P.; Okagu, O.D.; Udenigwe, C.C. Naturally Occurring Exosome Vesicles as Potential Delivery Vehicle for Bioactive Compounds. *Front. Sustain. Food Syst.* **2019**, *3*, 23. [CrossRef]
14. Morse, M.A.; Garst, J.; Osada, T.; Khan, S.; Hobeika, A.; Clay, T.M.; Valente, N.; Shreeniwas, R.; Sutton, M.A.; Delcayre, A.; et al. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J. Transl. Med.* **2005**, *3*, 9. [CrossRef] [PubMed]
15. Saleh, A.F.; Lázaro-Ibáñez, E.; Forsgard, M.A.; Shatnyeva, O.; Osteikoetxea, X.; Karlsson, F.; Heath, N.; Ingelsten, M.; Rose, J.; Harris, J.; et al. Extracellular Vesicles Induce Minimal Hepatotoxicity and Immunogenicity. *Nanoscale* **2019**, *11*, 6990–7001. [CrossRef] [PubMed]
16. Kamerkar, S.; Leng, C.; Burenkova, O.; Jang, S.C.; McCoy, C.; Zhang, K.; Dooley, K.; Kasera, S.; Zi, T.; Sisó, S.; et al. Exosome-mediated genetic reprogramming of tumor-associated macrophages by exoASO-STAT6 leads to potent monotherapy antitumor activity. *Sci. Adv.* **2022**, *8*, eabj7002. [CrossRef]
17. Gurung, S.; Perocheau, D.; Touramanidou, L.; Baruteau, J. The exosome journey: From biogenesis to uptake and intracellular signalling. *Cell Commun. Signal.* **2021**, *19*, 47. [CrossRef]
18. Konoshenko, M.Y.; Lekchnov, E.A.; Vlassov, A.V.; Laktionov, P.P. Isolation of Extracellular Vesicles: General Methodologies and Latest Trends. *BioMed Res. Int.* **2018**, *2018*, 8545347. [CrossRef]
19. Willms, E.; Johansson, H.J.; Mäger, I.; Lee, Y.; Blomberg, K.E.M.; Sadik, M.; Alaarg, A.; Smith, C.I.E.; Lehtiö, J.; EL Andaloussi, S.; et al. Cells release subpopulations of exosomes with distinct molecular and biological properties. *Sci. Rep.* **2016**, *6*, 22519. [CrossRef]
20. Zhang, B.; Wang, M.; Gong, A.; Zhang, X.; Wu, X.; Zhu, Y.; Shi, H.; Wu, L.; Zhu, W.; Qian, H.; et al. HucMSC-Exosome Mediated-Wnt4 Signaling Is Required for Cutaneous Wound Healing. *Stem Cells* **2015**, *33*, 2158–2168. [CrossRef]
21. Cui, X.; He, Z.; Liang, Z.; Chen, Z.; Wang, H.; Zhang, J. Exosomes From Adipose-derived Mesenchymal Stem Cells Protect the Myocardium Against Ischemia/Reperfusion Injury Through Wnt/β-Catenin Signaling Pathway. *J. Cardiovasc. Pharmacol.* **2017**, *70*, 225–231. [CrossRef] [PubMed]
22. Gangadaran, P.; Madhyastha, H.; Madhyastha, R.; Rajendran, R.L.; Nakajima, Y.; Watanabe, N.; Velikkakath, A.K.G.; Hong, C.M.; Gopi, R.V.; Muthukalianan, G.K.; et al. The emerging role of exosomes in innate immunity, diagnosis and therapy. *Front. Immunol.* **2023**, *13*, 1085057. [CrossRef] [PubMed]
23. Liu, X.; Wei, Q.; Lu, L.; Cui, S.; Ma, K.; Zhang, W.; Ma, F.; Li, H.; Fu, X.; Zhang, C. Immunomodulatory potential of mesenchymal stem cell-derived extracellular vesicles: Targeting immune cells. *Front. Immunol.* **2023**, *14*, 1094685. [CrossRef] [PubMed]
24. Osaki, M.; Okada, F. Exosomes and Their Role in Cancer Progression. *Yonago Acta Med.* **2019**, *62*, 182–190. [CrossRef]
25. Bang, C.; Batkai, S.; Dangwal, S.; Gupta, S.K.; Foinquinos, A.; Holzmann, A.; Just, A.; Remke, J.; Zimmer, K.; Zeug, A.; et al. Cardiac fibroblast–derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy. *J. Clin. Investig.* **2014**, *124*, 2136–2146. [CrossRef]
26. Kalluri, R.; LeBleu, V.S. The Biology, Function, and Biomedical Applications of Exosomes. *Science* **2020**, *367*, eaau6977. [CrossRef]
27. Jeppesen, D.K.; Fenix, A.M.; Franklin, J.L.; Higginbotham, J.N.; Zhang, Q.; Zimmerman, L.J.; Liebler, D.C.; Ping, J.; Liu, Q.; Evans, R.; et al. Reassessment of Exosome Composition. *Cell* **2019**, *177*, 428–445.e18. [CrossRef]
28. Beer, K.B.; Wehman, A.M. Mechanisms and functions of extracellular vesicle release in vivo-What we can learn from flies and worms. *Cell Adhes. Migr.* **2017**, *11*, 135–150. [CrossRef]
29. Ståhl, A.; Johansson, K.; Mossberg, M.; Kahn, R.; Karpman, D. Exosomes and microvesicles in normal physiology, pathophysiology, and renal diseases. *Pediatr. Nephrol.* **2019**, *34*, 11–30. [CrossRef]
30. Poon, I.K.H.; Parkes, M.A.F.; Jiang, L.; Atkin-Smith, G.K.; Tixeira, R.; Gregory, C.D.; Ozkocak, D.C.; Rutter, S.F.; Caruso, S.; Santavanond, J.P.; et al. Moving beyond size and phosphatidylserine exposure: Evidence for a diversity of apoptotic cell-derived extracellular vesicles in vitro. *J. Extracell. Vesicles* **2019**, *8*, 1608786. [CrossRef]
31. Battistelli, M.; Falcieri, E. Apoptotic bodies: Particular extracellular vesicles involved in intercellular communication. *Biology* **2020**, *9*, 21. [CrossRef] [PubMed]
32. Osteikoetxea, X.; Balogh, A.; Szabó-Taylor, K.; Németh, A.; Szabó, T.G.; Pálóczi, K.; Sódar, B.; Kittel, Á.; György, B.; Pállinger, É.; et al. Improved Characterization of EV Preparations Based on Protein to Lipid Ratio and Lipid Properties. *PLoS ONE* **2015**, *10*, e0121184. [CrossRef]
33. Tan, S.S.; Yin, Y.; Lee, T.; Lai, R.C.; Yeo, R.W.Y.; Zhang, B.; Choo, A.; Lim, S.K. Therapeutic MSC exosomes are derived from lipid raft microdomains in the plasma membrane. *J. Extracell. Vesicles* **2013**, *2*, 22614. [CrossRef] [PubMed]
34. Garcia-Contreras, M.; Brooks, R.W.; Boccuzzi, L.; Robbins, P.D.; Ricordi, C. Exosomes as biomarkers and therapeutic tools for type 1 diabetes mellitus. *Eur. Rev. Med. Pharmacol. Sci.* **2017**, *21*, 2940–2956.
35. Mathivanan, S.; Simpson, R.J. ExoCarta: A compendium of exosomal proteins and RNA. *Proteomics* **2009**, *9*, 4997–5000. [CrossRef] [PubMed]

36. Teng, F.; Fussenegger, M. Shedding Light on Extracellular Vesicle Biogenesis and Bioengineering. *Adv. Sci.* **2021**, *8*, 2003505. [CrossRef] [PubMed]
37. Burtenshaw, D.; Regan, B.; Owen, K.; Collins, D.; McEneaney, D.; Megson, I.L.; Redmond, E.M.; Cahill, P.A. Exosomal Composition, Biogenesis and Profiling Using Point-of-Care Diagnostics—Implications for Cardiovascular Disease. *Front. Cell Dev. Biol.* **2022**, *10*, 853451. [CrossRef]
38. Cossetti, C.; Iraci, N.; Mercer, T.R.; Leonardi, T.; Alpi, E.; Drago, D.; Alfaro-Cervello, C.; Saini, H.K.; Davis, M.P.; Schaeffer, J.; et al. Extracellular vesicles from neural stem cells transfer IFN-γ via Ifngr1 to activate Stat1 signaling in target cells. *Mol. Cell* **2014**, *56*, 193–204. [CrossRef]
39. Buschow, S.I.; van Balkom, B.W.M.; Aalberts, M.; Heck, A.J.R.; Wauben, M.; Stoorvogel, W. MHC class II-associated proteins in B-cell exosomes and potential functional implications for exosome biogenesis. *Immunol. Cell Biol.* **2010**, *88*, 851–856. [CrossRef]
40. Zhang, H.; Deng, T.; Liu, R.; Bai, M.; Zhou, L.; Wang, X.; Li, S.; Wang, X.; Yang, H.; Li, J. Exosome-delivered EGFR regulates liver microenvironment to promote gastric cancer liver metastasis. *Nat. Commun.* **2017**, *8*, 15016. [CrossRef]
41. Koga, K.; Matsumoto, K.; Akiyoshi, T.; Kubo, M.; Yamanaka, N.; Tasaki, A.; Nakashima, H.; Nakamura, M.; Kuroki, S.; Tanaka, M.; et al. Purification, characterization and biological significance of tumor-derived exosomes. *Anticancer Res.* **2005**, *25*, 3703–3707. [PubMed]
42. Ruhen, O.; Qu, X.; Jamaluddin, M.F.B.; Salomon, C.; Gandhi, A.; Millward, M.; Nixon, B.; Dun, M.D.; Meehan, K. Dynamic Landscape of Extracellular Vesicle-Associated Proteins Is Related to Treatment Response of Patients with Metastatic Breast Cancer. *Membranes* **2021**, *11*, 880. [CrossRef] [PubMed]
43. Théry, C.; Witwer, K.W.; Aikawa, E.; Alcaraz, M.J.; Anderson, J.D.; Andriantsitohaina, R.; Antoniou, A.; Arab, T.; Archer, F.; Atkin-Smith, G.K.; et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* **2018**, *7*, 1535750. [CrossRef] [PubMed]
44. Oksvold, M.P.; Kullmann, A.; Forfang, L.; Kierulf, B.; Li, M.; Brech, A.; Vlassov, A.V.; Smeland, E.B.; Neurauter, A.; Pedersen, K.W. Expression of B-Cell Surface Antigens in Subpopulations of Exosomes Released from B-Cell Lymphoma Cells. *Clin. Ther.* **2014**, *36*, 847–862.e1. [CrossRef] [PubMed]
45. French, K.C.; Antonyak, M.A.; Cerione, R.A. Extracellular vesicle docking at the cellular port: Extracellular vesicle binding and uptake. *Semin. Cell Dev. Biol.* **2017**, *67*, 48–55. [CrossRef]
46. Rodrigues, P.; Melim, C.; Veiga, F.; Figueiras, A. An Overview of Exosomes in Cancer Therapy: A Small Solution to a Big Problem. *Processes* **2020**, *8*, 1561. [CrossRef]
47. Tenchov, R.; Sasso, J.M.; Wang, X.; Liaw, W.-S.; Chen, C.-A.; Zhou, Q.A. Exosomes–Nature's Lipid Nanoparticles, a Rising Star in Drug Delivery and Diagnostics. *ACS Nano* **2022**, *16*, 17802–17846. [CrossRef]
48. Yu, L.-L.; Zhu, J.; Liu, J.-X.; Jiang, F.; Ni, W.-K.; Qu, L.-S.; Ni, R.-Z.; Lu, C.-H.; Xiao, M.-B. A Comparison of Traditional and Novel Methods for the Separation of Exosomes from Human Samples. *BioMed Res. Int.* **2018**, *2018*, 3634563. [CrossRef]
49. Exosome Isolation Kit Pan, Human. Available online: https://www.miltenyibiotec.com/DE-en/products/exosome-isolation-kit-pan-human.html?countryRedirected=1#gref (accessed on 15 December 2022).
50. Li, P.; Kaslan, M.; Lee, S.H.; Yao, J.; Gao, Z. Progress in Exosome Isolation Techniques. *Theranostics* **2017**, *7*, 789–804. [CrossRef]
51. Szatanek, R.; Baran, J.; Siedlar, M.; Baj-Krzyworzeka, M. Isolation of extracellular vesicles: Determining the correct approach (Review). *Int. J. Mol. Med.* **2015**, *36*, 11–17. [CrossRef]
52. Deregibus, M.C.; Figliolini, F.; D'Antico, S.; Manzini, P.M.; Pasquino, C.; De Lena, M.; Tetta, C.; Brizzi, M.F.; Camussi, G. Charge-based precipitation of extracellular vesicles. *Int. J. Mol. Med.* **2016**, *38*, 1359–1366. [CrossRef] [PubMed]
53. Heath, N.; Grant, L.; De Oliveira, T.M.; Rowlinson, R.; Osteikoetxea, X.; Dekker, N.; Overman, R. Rapid isolation and enrichment of extracellular vesicle preparations using anion exchange chromatography. *Sci. Rep.* **2018**, *8*, 5730. [CrossRef] [PubMed]
54. Bachurski, D.; Schuldner, M.; Nguyen, P.H.; Malz, A.; Reiners, K.S.; Grenzi, P.C.; Babatz, F.; Schauss, A.C.; Hansen, H.P.; Hallek, M.; et al. Extracellular vesicle measurements with nanoparticle tracking analysis–An accuracy and repeatability comparison between NanoSight NS300 and ZetaView. *J. Extracell. Vesicles* **2019**, *8*, 1596016. [CrossRef] [PubMed]
55. Carnino, J.M.; Lee, H.; Jin, Y. Isolation and characterization of extracellular vesicles from Broncho-Alveolar lavage fluid: A review and comparison of different methods. *Respir. Res.* **2019**, *20*, 240. [CrossRef]
56. Hartjes, T.A.; Mytnyk, S.; Jenster, G.W.; Van Steijn, V.; Van Royen, M.E. Extracellular Vesicle Quantification and Characterization: Common Methods and Emerging Approaches. *Bioengineering* **2019**, *6*, 7. [CrossRef]
57. Zeta Potential—What Is It and How Can It Be Characterised? Available online: https://analytik.co.uk/zeta-potential-what-is-it-and-how-can-it-be-characterised/ (accessed on 11 January 2023).
58. Midekessa, G.; Godakumara, K.; Ord, J.; Viil, J.; Lättekivi, F.; Dissanayake, K.; Kopanchuk, S.; Rinken, A.; Andronowska, A.; Bhattacharjee, S.; et al. Zeta Potential of Extracellular Vesicles: Toward Understanding the Attributes that Determine Colloidal Stability. *ACS Omega* **2020**, *5*, 16701–16710. [CrossRef]
59. Clogston, J.D.; Patri, A.K. Zeta Potential Measurement. In *Characterization of Nanoparticles Intended for Drug Delivery*; Methods in Molecular Biology; Humana Press: New York, NY, USA, 2011; Volume 697, pp. 63–70. [CrossRef]
60. Sowerby, S.J.; Broom, M.F.; Petersen, G.B. Dynamically resizable nanometre-scale apertures for molecular sensing. *Sens. Actuators B Chem.* **2007**, *123*, 325–330. [CrossRef]

61. Pei, Y.; Vogel, R.; Minelli, C. Tunable resistive pulse sensing (TRPS). *Charact. Nanopart. Meas. Process. Nanopart.* **2020**, 117–136. [CrossRef]
62. Chen, C.; Gao, K.; Lian, H.; Chen, C.; Yan, X. Single-particle characterization of theranostic liposomes with stimulus sensing and controlled drug release properties. *Biosens. Bioelectron.* **2019**, *131*, 185–192. [CrossRef]
63. The NanoAnalyzer(EVs)-NanoFCM. Available online: https://www.nanofcm.com/ev-c/the-nanoanalyzer/ (accessed on 18 January 2023).
64. Morales-Kastresana, A.; Jones, J.C. Flow Cytometric Analysis of Extracellular Vesicles. In *Exosomes Microvesicles*; Methods in Molecular Biology; Humana Press: New York, NY, USA, 2017; Volume 1545, pp. 215–225. [CrossRef]
65. Suárez, H.; Gámez-Valero, A.; Reyes, R.; López-Martín, S.; Rodríguez, M.J.; Carrascosa, J.L.; Cabañas, C.; Borràs, F.E.; Yáñez-Mó, M. A bead-assisted flow cytometry method for the semi-quantitative analysis of Extracellular Vesicles. *Sci. Rep.* **2017**, *7*, 11271. [CrossRef]
66. Mahmood, T.; Yang, P.C. Western Blot: Technique, Theory, and Trouble Shooting. *N. Am. J. Med. Sci.* **2012**, *4*, 429. [CrossRef] [PubMed]
67. Silva, J.M.; McMahon, M. The Fastest Western in Town: A Contemporary Twist on the Classic Western Blot Analysis. *J. Vis. Exp. JoVE* **2014**, *84*, 51149. [CrossRef]
68. Höög, J.L.; Lötvall, J. Diversity of extracellular vesicles in human ejaculates revealed by cryo-electron microscopy. *J. Extracell. Vesicles* **2015**, *4*, 28680. [CrossRef] [PubMed]
69. Yuana, Y.; Koning, R.I.; Kuil, M.E.; Rensen, P.C.N.; Koster, A.J.; Bertina, R.M.; Osanto, S. Cryo-electron microscopy of extracellular vesicles in fresh plasma. *J. Extracell. Vesicles* **2013**, *2*, 21494. [CrossRef] [PubMed]
70. Raposo, G.; Stoorvogel, W. Extracellular vesicles: Exosomes, microvesicles, and friends. *J. Cell Biol.* **2013**, *200*, 373–383. [CrossRef] [PubMed]
71. Jung, M.K.; Mun, J.Y. Sample Preparation and Imaging of Exosomes by Transmission Electron Microscopy. *J. Vis. Exp. JoVE* **2018**, *2018*, 56482. [CrossRef]
72. Luan, X.; Sansanaphongpricha, K.; Myers, I.; Chen, H.; Yuan, H.; Sun, D. Engineering exosomes as refined biological nanoplatforms for drug delivery. *Acta Pharmacol. Sin.* **2017**, *38*, 754–763. [CrossRef]
73. Sun, D.; Zhuang, X.; Xiang, X.; Liu, Y.; Zhang, S.; Liu, C.; Barnes, S.; Grizzle, W.; Miller, D.; Zhang, H.-G. A novel nanoparticle drug delivery system: The anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Mol. Ther. J. Am. Soc. Gene Ther.* **2010**, *18*, 1606–1614. [CrossRef]
74. Haney, M.J.; Klyachko, N.L.; Zhao, Y.; Gupta, R.; Plotnikova, E.G.; He, Z.; Patel, T.; Piroyan, A.; Sokolsky, M.; Kabanov, A.V.; et al. Exosomes as drug delivery vehicles for Parkinson's disease therapy. *J. Control. Release Off. J. Control. Release Soc.* **2015**, *207*, 18–30. [CrossRef]
75. Haney, M.J.; Klyachko, N.L.; Zhao, Y.; Kabanov, A.V.; Batrakova, E.V. P22—Extracellular Vesicles as Drug Delivery Vehicles for Potent Redox Enzyme Catalase to Treat Parkinson's Disease. *Free Radic. Biol. Med.* **2018**, *128*, S18. [CrossRef]
76. Podolak, I.; Galanty, A.; Sobolewska, D. Saponins as cytotoxic agents: A review. *Phytochem. Rev. Proc. Phytochem. Soc. Eur.* **2010**, *9*, 425–474. [CrossRef] [PubMed]
77. Kooijmans, S.A.A.; Stremersch, S.; Braeckmans, K.; de Smedt, S.C.; Hendrix, A.; Wood, M.J.A.; Schiffelers, R.M.; Raemdonck, K.; Vader, P. Electroporation-induced siRNA precipitation obscures the efficiency of siRNA loading into extracellular vesicles. *J. Control. Release* **2013**, *172*, 229–238. [CrossRef] [PubMed]
78. Shen, B.; Wu, N.; Yang, J.-M.; Gould, S.J. Protein targeting to exosomes/microvesicles by plasma membrane anchors. *J. Biol. Chem.* **2011**, *286*, 14383–14395. [CrossRef] [PubMed]
79. Nakase, I.; Noguchi, K.; Aoki, A.; Takatani-Nakase, T.; Fujii, I.; Futaki, S. Arginine-rich cell-penetrating peptide-modified extracellular vesicles for active macropinocytosis induction and efficient intracellular delivery. *Sci. Rep.* **2017**, *7*, 1991. [CrossRef] [PubMed]
80. Munagala, R.; Aqil, F.; Jeyabalan, J.; Gupta, R.C. Bovine milk-derived exosomes for drug delivery. *Cancer Lett.* **2016**, *371*, 48–61. [CrossRef]
81. Lin, Q.; Qu, M.; Zhou, B.; Patra, H.K.; Sun, Z.; Luo, Q.; Yang, W.; Wu, Y.; Zhang, Y.; Li, L.; et al. Exosome-like nanoplatform modified with targeting ligand improves anti-cancer and anti-inflammation effects of imperialine. *J. Control. Release* **2019**, *311–312*, 104–116. [CrossRef]
82. Surana, R.; LeBleu, V.S.; Lee, J.J.; Smaglo, B.G.; Zhao, D.; Lee, M.S.; Wolff, R.A.; Overman, M.J.; Mendt, M.C.; McAndrews, K.M.; et al. Phase I study of mesenchymal stem cell (MSC)-derived exosomes with KRASG12D siRNA in patients with metastatic pancreatic cancer harboring a KRASG12D mutation. *J. Clin. Oncol.* **2022**, *40*, TPS633. [CrossRef]
83. Lewis, N.D.; Sia, C.L.; Kirwin, K.; Haupt, S.; Mahimkar, G.; Zi, T.; Xu, K.; Dooley, K.; Jang, S.C.; Choi, B.; et al. Exosome surface display of IL12 results in tumor-retained pharmacology with superior potency and limited systemic exposure compared with recombinant IL12. *Mol. Cancer Ther.* **2021**, *20*, 523–534. [CrossRef]
84. Jang, S.C.; Economides, K.D.; Moniz, R.J.; Sia, C.L.; Lewis, N.; McCoy, C.; Zi, T.; Zhang, K.; Harrison, R.A.; Lim, J.; et al. ExoSTING, an extracellular vesicle loaded with STING agonists, promotes tumor immune surveillance. *Commun. Biol.* **2021**, *4*, 497. [CrossRef]
85. Zhang, M.; Viennois, E.; Prasad, M.; Zhang, Y.; Wang, L.; Zhang, Z.; Han, M.K.; Xiao, B.; Xu, C.; Srinivasan, S.; et al. Edible ginger-derived nanoparticles: A novel therapeutic approach for the prevention and treatment of inflammatory bowel disease and colitis-associated cancer. *Biomaterials* **2016**, *101*, 321–340. [CrossRef]

86. Johnsen, K.B.; Gudbergsson, J.M.; Skov, M.N.; Pilgaard, L.; Moos, T.; Duroux, M. A comprehensive overview of exosomes as drug delivery vehicles — Endogenous nanocarriers for targeted cancer therapy. *Biochim. Biophys. Acta BBA Rev. Cancer* **2014**, *1846*, 75–87. [CrossRef] [PubMed]
87. Dong, S.; Liu, X.; Bi, Y.; Wang, Y.; Antony, A.; Lee, D.; Huntoon, K.; Jeong, S.; Ma, Y.; Li, X.; et al. Adaptive design of mRNA-loaded extracellular vesicles for targeted immunotherapy of cancer. *Nat. Commun.* **2023**, *14*, 6610. [CrossRef] [PubMed]
88. Yoo, M.H.; Lee, A.-R.; Moon, K.-S. Characteristics of Extracellular Vesicles and Preclinical Testing Considerations Prior to Clinical Applications. *Biomedicines* **2022**, *10*, 869. [CrossRef] [PubMed]
89. Briuglia, M.-L.; Rotella, C.; McFarlane, A.; Lamprou, D.A. Influence of cholesterol on liposome stability and on in vitro drug release. *Drug Deliv. Transl. Res.* **2015**, *5*, 231–242. [CrossRef] [PubMed]
90. Kirby, C.; Clarke, J.; Gregoriadis, G. Effect of the Cholesterol Content of Small Unilamellar Liposomes on their Stability in vivo and in vitro. *Biochem. J.* **1980**, *186*, 591–598. [CrossRef]
91. Rohner, E.; Yang, R.; Foo, K.S.; Goedel, A.; Chien, K.R. Unlocking the promise of mRNA therapeutics. *Nat. Biotechnol.* **2022**, *40*, 1586–1600. [CrossRef]
92. Coelho, T.; Adams, D.; Silva, A.; Lozeron, P.; Hawkins, P.N.; Mant, T.; Perez, J.; Chiesa, J.; Warrington, S.; Tranter, E.; et al. Safety and Efficacy of RNAi Therapy for Transthyretin Amyloidosis. *N. Engl. J. Med.* **2013**, *369*, 819–829. [CrossRef]
93. Adams, D.; Gonzalez-Duarte, A.; O'Riordan, W.D.; Yang, C.-C.; Ueda, M.; Kristen, A.V.; Tournev, I.; Schmidt, H.H.; Coelho, T.; Berk, J.L.; et al. Patisiran, an RNAi Therapeutic, for Hereditary Transthyretin Amyloidosis. *N. Engl. J. Med.* **2018**, *379*, 11–21. [CrossRef]
94. Han, X.; Zhang, H.; Butowska, K.; Swingle, K.L.; Alameh, M.-G.; Weissman, D.; Mitchell, M.J. An ionizable lipid toolbox for RNA delivery. *Nat. Commun.* **2021**, *12*, 7233. [CrossRef]
95. Maier, M.A.; Jayaraman, M.; Matsuda, S.; Liu, J.; Barros, S.; Querbes, W.; Tam, Y.K.; Ansell, S.M.; Kumar, V.; Qin, J.; et al. Biodegradable lipids enabling rapidly eliminated lipid nanoparticles for systemic delivery of RNAi therapeutics. *Mol. Ther. J. Am. Soc. Gene Ther.* **2013**, *21*, 1570–1578. [CrossRef]
96. Mendt, M.; Kamerkar, S.; Sugimoto, H.; McAndrews, K.M.; Wu, C.-C.; Gagea, M.; Yang, S.; Blanko, E.V.R.; Peng, Q.; Ma, X.; et al. Generation and testing of clinical-grade exosomes for pancreatic cancer. *JCI Insight* **2018**, *3*, e99263. [CrossRef] [PubMed]
97. Silva, A.K.A.; Luciani, N.; Gazeau, F.; Aubertin, K.; Bonneau, S.; Chauvierre, C.; Letourneur, D.; Wilhelm, C. Combining magnetic nanoparticles with cell derived microvesicles for drug loading and targeting. *Nanomed. Nanotechnol. Biol. Med.* **2015**, *11*, 645–655. [CrossRef] [PubMed]
98. Sato, Y.; Umezaki, K.; Sawada, S.; Mukai, S.; Sasaki, Y.; Harada, N.; Shiku, H.; Akiyoshi, K. Engineering hybrid exosomes by membrane fusion with liposomes. *Sci. Rep.* **2016**, *6*, 21933. [CrossRef] [PubMed]
99. Joel, D.B. Hybridosomes, Compositions Comprising the Same, Processes for their Production and Uses Thereof. U.S. Patent 2016354313-A1, 18 February 2020.
100. Evers, M.J.W.; van de Wakker, S.I.; de Groot, E.M.; de Jong, O.G.; Gitz-François, J.J.J.; Seinen, C.S.; Sluijter, J.P.G.; Schiffelers, R.M.; Vader, P. Functional siRNA Delivery by Extracellular Vesicle–Liposome Hybrid Nanoparticles. *Adv. Healthc. Mater.* **2022**, *11*, 2101202. [CrossRef] [PubMed]
101. Zhu, Y.; Zhu, L.; Wang, X.; Jin, H. RNA-based therapeutics: An overview and prospectus. *Cell Death Dis.* **2022**, *13*, 644. [CrossRef]
102. Zamore, P.D.; Haley, B. Ribo-gnome: The Big World of Small RNAs. *Science* **2005**, *309*, 1519–1524. [CrossRef]
103. Yang, J.; Zhang, X.; Chen, X.; Wang, L.; Yang, G. Exosome Mediated Delivery of miR-124 Promotes Neurogenesis after Ischemia. *Mol. Ther. Nucleic Acids* **2017**, *7*, 278–287. [CrossRef]
104. Villarroya-Beltri, C.; Gutiérrez-Vázquez, C.; Sánchez-Cabo, F.; Pérez-Hernández, D.; Vázquez, J.; Martin-Cofreces, N.; Martinez-Herrera, D.J.; Pascual-Montano, A.; Mittelbrunn, M.; Sánchez-Madrid, F. Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. *Nat. Commun.* **2013**, *4*, 2980. [CrossRef]
105. Santangelo, L.; Giurato, G.; Cicchini, C.; Montaldo, C.; Mancone, C.; Tarallo, R.; Battistelli, C.; Alonzi, T.; Weisz, A.; Tripodi, M. The RNA-Binding Protein SYNCRIP Is a Component of the Hepatocyte Exosomal Machinery Controlling MicroRNA Sorting. *Cell Rep.* **2016**, *17*, 799–808. [CrossRef]
106. Hung, M.E.; Leonard, J.N. A platform for actively loading cargo RNA to elucidate limiting steps in EV-mediated delivery. *J. Extracell. Vesicles* **2016**, *5*, 31027. [CrossRef]
107. Bolukbasi, M.F.; Mizrak, A.; Ozdener, G.B.; Madlener, S.; Ströbel, T.; Erkan, E.P.; Fan, J.-B.; Breakefield, X.O.; Saydam, O. miR-1289 and "Zipcode"-like Sequence Enrich mRNAs in Microvesicles. *Mol. Ther. Nucleic Acids* **2012**, *1*, e10. [CrossRef] [PubMed]
108. Villarroya-Beltri, C.; Baixauli, F.; Gutiérrez-Vázquez, C.; Sánchez-Madrid, F.; Mittelbrunn, M. Sorting it out: Regulation of exosome loading. *Semin. Cancer Biol.* **2014**, *28*, 3–13. [CrossRef]
109. Usman, W.M.; Pham, T.C.; Kwok, Y.Y.; Vu, L.T.; Ma, V.; Peng, B.; Chan, Y.S.; Wei, L.; Chin, S.M.; Azad, A.; et al. Efficient RNA drug delivery using red blood cell extracellular vesicles. *Nat. Commun.* **2018**, *9*, 2359. [CrossRef] [PubMed]
110. Prada, I.; Meldolesi, J. Binding and Fusion of Extracellular Vesicles to the Plasma Membrane of Their Cell Targets. *Int. J. Mol. Sci.* **2016**, *17*, 1296. [CrossRef] [PubMed]
111. O'Brien, K.; Breyne, K.; Ughetto, S.; Laurent, L.C.; Breakefield, X.O. RNA delivery by extracellular vesicles in mammalian cells and its applications. *Nat. Rev. Mol. Cell Biol.* **2020**, *21*, 585–606. [CrossRef]
112. Toribio, V.; Morales, S.; López-Martín, S.; Cardeñes, B.; Cabañas, C.; Yáñez-Mó, M. Development of a quantitative method to measure EV uptake. *Sci. Rep.* **2019**, *9*, 10522. [CrossRef]

113. Quail, D.F.; Joyce, J.A. The microenvironmental landscape of brain tumors. *Cancer Cell* **2017**, *31*, 326–341. [CrossRef]
114. Didiot, M.-C.; Hall, L.M.; Coles, A.H.; Haraszti, R.A.; Godinho, B.M.; Chase, K.; Sapp, E.; Ly, S.; Alterman, J.F.; Hassler, M.R.; et al. Exosome-mediated Delivery of Hydrophobically Modified siRNA for Huntingtin mRNA Silencing. *Mol. Ther.* **2016**, *24*, 1836–1847. [CrossRef]
115. Kordasiewicz, H.B.; Stanek, L.M.; Wancewicz, E.V.; Mazur, C.; McAlonis, M.M.; Pytel, K.A.; Artates, J.W.; Weiss, A.; Cheng, S.H.; Shihabuddin, L.S.; et al. Sustained Therapeutic Reversal of Huntington's Disease by Transient Repression of Huntingtin Synthesis. *Neuron* **2012**, *74*, 1031–1044. [CrossRef]
116. Huang, R.; Du, H.; Cheng, L.; Zhang, P.; Meng, F.; Zhong, Z. Targeted nanodelivery of siRNA against KRAS G12D inhibits pancreatic cancer. *Acta Biomater.* **2023**, *168*, 529–539. [CrossRef]
117. Paramasivam, P.; Franke, C.; Stöter, M.; Höijer, A.; Bartesaghi, S.; Sabirsh, A.; Lindfors, L.; Yanez Arteta, M.; Dahlén, A.; Bak, A.; et al. Endosomal escape of delivered mRNA from endosomal recycling tubules visualized at the nanoscale. *J. Cell Biol.* **2021**, *221*, e202110137. [CrossRef] [PubMed]
118. Hassett, K.J.; Higgins, J.; Woods, A.; Levy, B.; Xia, Y.; Hsiao, C.J.; Acosta, E.; Almarsson, Ö.; Moore, M.J.; Brito, L.A. Impact of lipid nanoparticle size on mRNA vaccine immunogenicity. *J. Control. Release* **2021**, *335*, 237–246. [CrossRef] [PubMed]
119. Ribovski, L.; Joshi, B.S.; Gao, J.; Zuhorn, I.S. Breaking free: Endocytosis and endosomal escape of extracellular vesicles. *Extracell. Vesicles Circ. Nucleic Acids* **2023**, *4*, 283–305. [CrossRef]
120. Elsharkasy, O.M.; Nordin, J.Z.; Hagey, D.W.; de Jong, O.G.; Schiffelers, R.M.; Andaloussi, S.E.; Vader, P. Extracellular vesicles as drug delivery systems: Why and how? *Adv. Drug Deliv. Rev.* **2020**, *159*, 332–343. [CrossRef] [PubMed]
121. Zubarev, I.; Vladimirtsev, D.; Vorontsova, M.; Blatov, I.; Shevchenko, K.; Zvereva, S.; Lunev, E.A.; Faizuloev, E.; Barlev, N. Viral Membrane Fusion Proteins and RNA Sorting Mechanisms for the Molecular Delivery by Exosomes. *Cells* **2021**, *10*, 3043. [CrossRef]
122. Hou, X.; Zaks, T.; Langer, R.; Dong, Y. Lipid nanoparticles for mRNA delivery. *Nat. Rev. Mater.* **2021**, *6*, 1078–1094. [CrossRef]
123. El-Andaloussi, S.; Lee, Y.; Lakhal-Littleton, S.; Li, J.; Seow, Y.; Gardiner, C.; Alvarez-Erviti, L.; Sargent, I.L.; Wood, M.J.A. Exosome-mediated delivery of siRNA in vitro and in vivo. *Nat. Protoc.* **2012**, *7*, 2112–2126. [CrossRef]
124. Todaro, B.; Santi, M. Characterization and Functionalization Approaches for the Study of Polymeric Nanoparticles: The State of the Art in Italian Research. *Micro* **2023**, *3*, 9–21. [CrossRef]
125. Roy, A.; Nair, S.; Sen, N.; Soni, N.; Madhusudhan, M.S. In silico methods for design of biological therapeutics. *Methods* **2017**, *131*, 33–65. [CrossRef]
126. Lee, A.C.-L.; Harris, J.L.; Khanna, K.K.; Hong, J.-H. A Comprehensive Review on Current Advances in Peptide Drug Development and Design. *Int. J. Mol. Sci.* **2019**, *20*, 2383. [CrossRef]
127. Raha, S.; Paunesku, T.; Woloschak, G. Peptide-mediated cancer targeting of nanoconjugates. *WIREs Nanomed. Nanobiotechnol.* **2011**, *3*, 269–281. [CrossRef] [PubMed]
128. Alvarez-Erviti, L.; Seow, Y.; Yin, H.; Betts, C.; Lakhal, S.; Wood, M.J.A. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat. Biotechnol.* **2011**, *29*, 341–345. [CrossRef] [PubMed]
129. Yang, Y.; Hong, Y.; Cho, E.; Kim, G.B.; Kim, I.-S. Extracellular vesicles as a platform for membrane-associated therapeutic protein delivery. *J. Extracell. Vesicles* **2018**, *7*, 1440131. [CrossRef]
130. Ivanova, A.; Kohl, F.; Garibotti, H.G.-K.; Chalupska, R.; Cvjetkovic, A.; Firth, M.; Jennbacken, K.; Martinsson, S.; Silva, A.M.; Viken, I.; et al. In vivo phage display identifies novel peptides for cardiac targeting. *Sci. Rep.* **2024**, *14*, 12177. [CrossRef] [PubMed]
131. Zhou, Y.; Yuan, Y.; Liu, M.; Hu, X.; Quan, Y.; Chen, X. Tumor-specific delivery of KRAS siRNA with iRGD-exosomes efficiently inhibits tumor growth. *ExRNA* **2019**, *1*, 28. [CrossRef]
132. Bai, J.; Duan, J.; Liu, R.; Du, Y.; Luo, Q.; Cui, Y.; Su, Z.; Xu, J.; Xie, Y.; Lu, W. Engineered targeting tLyp-1 exosomes as gene therapy vectors for efficient delivery of siRNA into lung cancer cells. *Asian J. Pharm. Sci.* **2020**, *15*, 461–471. [CrossRef]
133. Smyth, T.; Petrova, K.; Payton, N.M.; Persaud, I.; Redzic, J.S.; Graner, M.W.; Smith-Jones, P.; Anchordoquy, T.J. Surface Functionalization of Exosomes Using Click Chemistry. *Bioconjug. Chem.* **2014**, *25*, 1777–1784. [CrossRef]
134. Jia, G.; Han, Y.; An, Y.; Ding, Y.; He, C.; Wang, X.; Tang, Q. NRP-1 targeted and cargo-loaded exosomes facilitate simultaneous imaging and therapy of glioma in vitro and in vivo. *Biomaterials* **2018**, *178*, 302–316. [CrossRef]
135. Tian, T.; Zhang, H.-X.; He, C.-P.; Fan, S.; Zhu, Y.-L.; Qi, C.; Huang, N.-P.; Xiao, Z.-D.; Lu, Z.-H.; Tannous, B.A.; et al. Surface functionalized exosomes as targeted drug delivery vehicles for cerebral ischemia therapy. *Biomaterials* **2018**, *150*, 137–149. [CrossRef]
136. Koh, E.; Lee, E.J.; Nam, G.-H.; Hong, Y.; Cho, E.; Yang, Y.; Kim, I.-S. Exosome-SIRPα, a CD47 blockade increases cancer cell phagocytosis. *Biomaterials* **2017**, *121*, 121–129. [CrossRef]
137. Nie, W.; Wu, G.; Zhang, J.; Huang, L.L.; Ding, J.; Jiang, A.; Zhang, Y.; Liu, Y.; Li, J.; Pu, K.; et al. Responsive exosome nano-bioconjugates for synergistic cancer therapy. *Angew. Chem. Int. Ed. Engl.* **2019**, *59*, 2018–2022. [CrossRef] [PubMed]
138. Cao, Y.; Wu, T.; Zhang, K.; Meng, X.; Dai, W.; Wang, D.; Dong, H.; Zhang, X. Engineered Exosome-Mediated Near-Infrared-II Region V2C Quantum Dot Delivery for Nucleus-Target Low-Temperature Photothermal Therapy. *ACS Nano* **2019**, *13*, 1499–1510. [CrossRef] [PubMed]
139. Kim, M.S.; Haney, M.J.; Zhao, Y.; Yuan, D.; Deygen, I.; Klyachko, N.L.; Kabanov, A.V.; Batrakova, E.V. Engineering macrophage-derived exosomes for targeted paclitaxel delivery to pulmonary metastases: In vitro and in vivo evaluations. *Nanomed. Nanotechnol. Biol. Med.* **2018**, *14*, 195–204. [CrossRef] [PubMed]

140. Pi, F.; Binzel, D.W.; Lee, T.J.; Li, Z.; Sun, M.; Rychahou, P.; Li, H.; Haque, F.; Wang, S.; Croce, C.M.; et al. Nanoparticle orientation to control RNA loading and ligand display on extracellular vesicles for cancer regression. *Nat. Nanotechnol.* **2018**, *13*, 82–89. [CrossRef] [PubMed]
141. Zou, J.; Shi, M.; Liu, X.; Jin, C.; Xing, X.; Qiu, L.; Tan, W. Aptamer-Functionalized Exosomes: Elucidating the Cellular Uptake Mechanism and the Potential for Cancer-Targeted Chemotherapy. *Anal. Chem.* **2019**, *91*, 2425–2430. [CrossRef]
142. Rezaie, J.; Feghhi, M.; Etemadi, T. A review on exosomes application in clinical trials: Perspective, questions, and challenges. *Cell Commun. Signal.* **2022**, *20*, 145. [CrossRef] [PubMed]
143. Han, L.; Zhao, Z.; He, C.; Li, J.; Li, X.; Lu, M. Removing the stumbling block of exosome applications in clinical and translational medicine: Expand production and improve accuracy. *Stem Cell Res. Ther.* **2023**, *14*, 57. [CrossRef]
144. Hussen, B.M.; Faraj, G.S.H.; Rasul, M.F.; Hidayat, H.J.; Salihi, A.; Baniahmad, A.; Taheri, M.; Ghafouri-Frad, S. Strategies to overcome the main challenges of the use of exosomes as drug carrier for cancer therapy. *Cancer Cell Int.* **2022**, *22*, 323. [CrossRef]
145. Chen, H.; Wang, L.; Zeng, X.; Schwarz, H.; Nanda, H.S.; Peng, X.; Zhou, Y. Exosomes, a New Star for Targeted Delivery. *Front. Cell Dev. Biol.* **2021**, *9*, 751079. [CrossRef]
146. Yin, Q.; Ji, X.; Lv, R.; Pei, J.-J.; Du, Y.; Shen, C.; Hou, X. Targetting Exosomes as a New Biomarker and Therapeutic Approach for Alzheimer's Disease. *Clin. Interv. Aging* **2020**, *15*, 195–205. [CrossRef]