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Review

Exosomes as Anticancer Drug Delivery Vehicles: Prospects and Challenges

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Abstract

Exosomes, a subset of extracellular vesicles, are widely present in various body fluids and are involved in mediating intercellular communication. They have received extensive attention as diagnostic markers. The excellent physicochemical and biological properties of exosomes make them great potential drug delivery vehicles for the treatment of cancer and other diseases. However, various challenges need to be addressed for the clinical application of exosomes. This review introduces the biogenesis and uptake of exosomes and compares different approaches for isolation and drug loading, focusing on the application and current challenges of exosomes as drug delivery vehicles in cancer therapy.

Keywords: exosomes; drug delivery; cancer

1. Introduction

Exosomes are tiny bilayer vesicles between 50 and 150 nm in diameter that are secreted by almost all cells [1]. In 1981, Tram and colleagues proposed exosomes as a name for exfoliated membrane vesicles discovered in the supernatant of *in vitro* cultured sheep erythrocytes [2]. In 1987, Johnstone and colleagues officially named these vesicles exosomes [3]. In earlier studies, exosomes were considered cellular waste disposal devices that only package and secrete discarded cellular contents into the extracellular matrix [4]. Growing evidence suggests that exosomes play a critical role in intercellular communication by transporting DNA, RNA, protein, microRNA (miRNA), and metabolites, which affect the life process of recipient cells [5]. They also play essential roles in physiological or pathological processes such as the regulation of immune responses, the occurrence of metabolic and neurodegenerative diseases, reproduction, and the development of mammals [6]. Exosomes are also involved in tumor development and progression by modulating the extracellular matrix in the tumor microenvironment, metastasis, and antitumor or protumor immune responses [7].

Exosomes are present in all biological fluids including cerebrospinal fluid, blood, saliva, urine, amniotic fluid, ascites, and bronchoalveolar lavage fluid [6,8,9]. Exosomes transport various cargoes including proteins, lipids, various RNA species, and DNA [4]. Since the types and amounts of cargoes vary in different diseases [1,10,11], exosomes are widely used for diagnosing and monitoring conditions [1,10]. Based on their biological functions and properties, inherently biologically active exosomes are a versatile drug delivery platform to treat cancer and other diseases by delivering genetic material (miRNA, spherical nucleic acids, and small interfering RNA [siRNA]), proteins, small molecule drugs (e.g., curcumin (cur), dopamine, paclitaxel (PTX), and doxorubicin), and other compounds that are stable in exosomes [11,12]. As natural nanoparticle biological carriers, exosomes are stable, membrane-permeable, cell-specific, and even able to penetrate the blood-brain barrier (BBB) [13,14]. Compared with other biological carriers, exosome-based therapeutic delivery has better efficacy and lower off-target effects [13,15].

However, some of the shortcomings of drug delivery vehicles are their low yield, heterogeneity, short half-life, and low loading efficiency of exosomes, which limit their clinical application [16,17]. As a result, new exosome preparation and drug-loading methods have been developed to promote the application of exosomes in cancer therapy.

There have been many excellent reviews and studies on exosomes as drug delivery vehicles [14,15,18,19]. This review focuses on advances in the field of cancer treatment; comprehensively and systematically summarizing exosome isolation and drug-loading methods; discusses their application and challenges in cancer treatment; and introduces the "source of exosomes", which has not received much attention.

2. Exosome Biogenesis

Exosomes are generated by the inward budding of the outer cell membrane [20]. Invagination of the cytoplasmic membrane forms early sorting endosomes (ESEs) that mature into late sorting endosomes. By the inward budding of

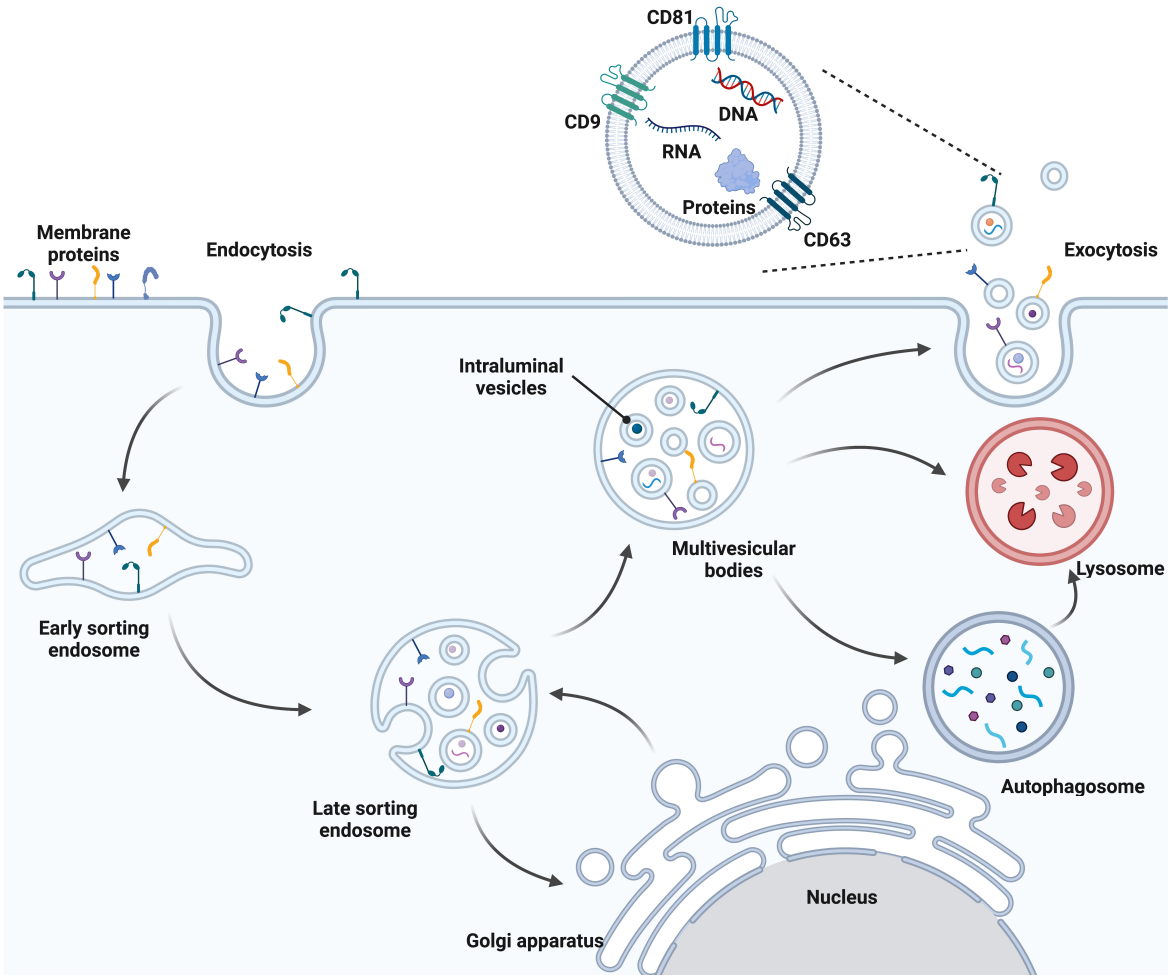

Fig. 1. Biogenesis of exosomes. Exosomes originate from endosomal structures and begin with invagination of the plasma membrane to form early sorting endosomes (ESEs). ESEs mature into late sorting endosomes, which exchange cargo with the Golgi and then bud inwards to develop multivesicular bodies (MVBs) that encapsulate intraluminal vesicle (ILVs), at this stage packaging exosomal contents. MVB can fuse with lysosomes or autophagosomes to be degraded or connect with the plasma membrane to release the contained ILV as exosomes. Exosomes contain various biologically active molecules including proteins and nucleic acids. The tetraspanins CD9, CD63, and CD81 on the exosome membrane are commonly used exosome marker proteins.

the late endosome membrane, intraluminal vesicle (ILV)-encapsulated biomolecules are generated in multivesicular bodies (MVBs) [21]. MVBs can then fuse with lysosomes or autophagosomes for degradation, or the plasma membrane to release the enclosed ILVs as exosomes [6]. Exosome biogenesis, however, can also occur via plasma membrane budding [22] (Fig. 1).

Exosome release and cargo sorting (such as lipids and ubiquitinated proteins) are regulated by several sorting processes. The most well-studied sorting system for exosome release and cargo sorting is the endosomal sorting complex (ESCRT). ESCRTs consist of approximately 20 proteins, which are assembled into four complexes (ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III) with related proteins such as vacuolar protein sorting-associated protein 4, vacuolar protein sorting-associated protein, and apoptosis-related gene 2-interacting protein X (ALIX) [23]. The ESCRT-0 complex mediates the recognition and sequestration of ubiquitinated proteins in the endosomal membrane [23]. Then the ESCRT-0 ubiquitin domain is recruited to the membrane by ESCRT-I and ESCRT-II, where the cargo is enclosed [24]. Finally, the projections are cleaved by ESCRT-III subunits to form ILVs [25]. Another ESCRT-independent protein sorting pathway also exists in MVBs [26]. For example, the tetraspanin cluster of differentiation 63 (CD63) participates in ESCRT-independent sorting of premelanosome protein into the LVs of MVBs [27]. Similarly, sphingolipid ceramides translocate exosome-associated domains to the endosomal lumen independently of the ESCRT [28].

In addition, the components involved in the origin and biogenesis of exosomes include the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, ALIX, syndecan-1, sytenin-1, Ras-

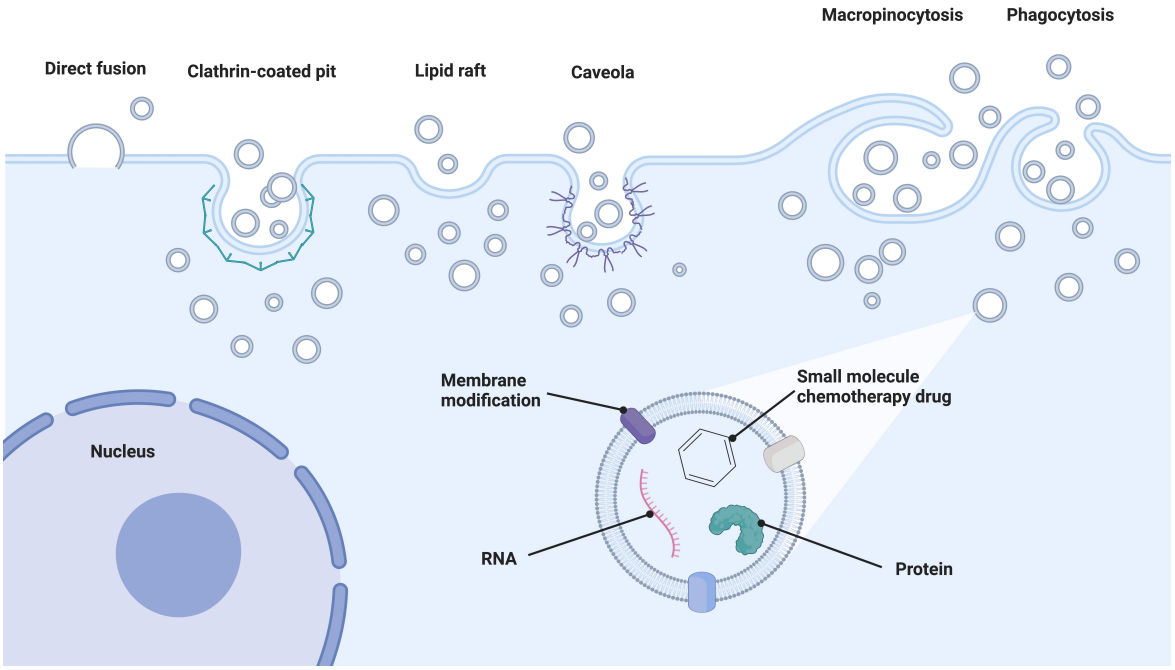

Fig. 2. Uptake of exosomes. Exosomes can enter cells through caveolin-dependent endocytosis, clathrin-dependent endocytosis, macropinocytosis, lipid rafts, phagocytosis, and direct fusion. Also, exosomes can enter cells through more than one route.

associated binding (Rab) protein GTPase, phospholipids, and sphingomyelinase [6]. Among them, small GTPase members of the Rab family play a well-established role in transferring vesicles between intracellular compartments and are also involved in transporting MVBs to the plasma membrane for exosome secretion. The SNARE complex is also required for MVB fusion with the plasma membrane [29]. The endosomal syndecan-syntenin complex interacts with ALIX to promote endosomal budding, which induces exosome biogenesis [30]. Phospholipids and sphingomyelinases play a role in exosome biogenesis and release by affecting ceramide synthesis [28].

3. Exosome Uptake

Exosome uptake can be mediated by endocytosis, including clathrin-dependent endocytosis, caveolae-dependent endocytosis, macropinocytosis, and phagocytosis [31] (Fig. 2). Notably, exosomes appear to enter cells via more than one route [32].

The interaction between receptors in the membrane of target cells and proteins of exosome surfaces are required for internalization, which is confirmed by the observation that the uptake efficiency was significantly reduced by proteinase K treatment [33]. Many exosomal proteins interact with receptors on target cells to mediate the cellular uptake of exosomes. For example, CD29/CD81 complex formation induced by radiation can increase the cellular uptake of exosomes [34]. Similarly, the uptake of exosomes by dendritic cells (DCs) is reduced after treatment of recipient cells with a CD9 antibody against tetraspanin [35]. In addition, integrins (integrins αv and β3, i.e., CD51 and CD61), proteoglycans (heparan sulfate proteoglycans), and lectins (C-type lectins DEC-205, galectin-5) affect the uptake or binding of exosomes by specific protein-protein interactions. Notably, while a growing number of specific protein interactions have been identified that mediate the attachment and uptake of exosomes, the underlying mechanisms of exosome-cell interactions require further investigation [32,36].

Clathrin-dependent endocytosis enables cellular internalization of molecules by assembling clathrin-coated vesicles through some membrane receptors and their ligands. The clathrin-coated blisters can distort the plasma membrane and collapse into mature and shed vesicle buds. The clathrin is then uncoated, and the vesicles fuse with the endosome, releasing the contents [32]. Chlorpromazine can inhibit this process [37]. Dynamin 2 is a GTPase required for the clathrin-dependent endocytosis process, which promotes membrane fusion, membrane curvature, membrane fission, and the release of clathrin-coated vesicles [32]. Exosome uptake has been observed in dynamin-positive cells, whereas exosome internalization is almost completely blocked in dynamin-negative cells [32,38]. Clathrin-dependent endocytosis is also involved with macropinocytosis in the uptake of PC12 cell-derived exosomes [39].

A parallel to clathrin-dependent endocytosis is cellular internalization through caveolae invagination, which is regulated by the same molecular machinery during fission [40]. Caveolae is a subdomain of plasma membrane glycolipid rafts rich in cholesterol, sphingolipids, and caveolin [32]. Caveolin-1 is necessary or sufficient for caveolae biogene-

sis; similarly, dynamin 2 is required for caveolin-dependent endocytosis [41]. Both dynamin inhibitor treatment and caveolin-1 knockdown significantly inhibit the internalization of exosomes via caveolae-dependent endocytosis [42].

Macropinocytosis typically occurs in the highly folded regions of the plasma membrane, leading to the uptake of extracellular solutes and fluids into 0.2- to 10-μm diameter vesicles or macropinosomes [41,43]. Some exosomes can be taken up into cells through macropinocytosis. For example, oligodendrocyte-derived exosomes can be transferred into microglia via micropinocytosis [44]. Similarly, macropinocytosis has been observed during exosome uptake in PC12 rat pheochromocytoma cells [39].

Furthermore, in addition to "professional" phagocytes such as DCs and macrophages, "non-professional" phagocytes such as HEK293T and Jurkat T cells can also internalize exosomes through phagocytosis [45]. This process relies on the actin cytoskeleton, dynamin 2, and phosphoinositide 3-kinase (PI3K) [38]. However, it is unclear whether phagocytosis is a means of exosome internalization for intercellular communication or only a method of exosome elimination; thus further investigation is required [45].

In addition, there is also a possible entry mechanism through direct fusion of the exosomal membrane with the plasma membrane, which is affected by the acidity of the microenvironment [46]. Membrane fusion is usually accomplished in two steps. First, the exosomal and plasma membranes are brought close to each other, and this process needs to overcome the counteracting electrostatic force. Second, because of the unstable interface between the hydrophilic and hydrophobic parts of the membrane, a transition state is formed, eventually resulting in hydrophilic fusion pores that facilitate fusion of the hydrophobic elements [47].

4. The Sources of Exosomes

As mentioned earlier, exosomes are present in all body fluids in the human body [6]. However, the yield of exosomes isolated from body fluids or culture media is insufficient to supply clinical demands. Product quality control is also a problem that limits its clinical application [48]. Exosomes from non-human sources such as bacteria, milk, and plants are considered a promising alternative for cancer therapy. These exosomes also have the potential to be used as personalized oral delivery vehicles due to their low toxicity and easy availability [18,49]. For example, exosome-like nanoparticles loaded with lemon-derived natural products can inhibit cancer cell proliferation by inducing tumor necrosis factor-related apoptosis-inducing ligand-mediated cell death [50]. Similarly, exosomes isolated from grapes, grapefruit, ginger, and carrots have also shown potential as drug delivery vehicles [51].

Milk-derived exosomes are also considered good delivery vehicles for hydrophilic and lipophilic drugs, including chemotherapeutic drugs, without showing systemic toxicity and immunogenicity in mice [48,52]. Moreover, experiments have demonstrated that unloaded milk exosomes significantly inhibit lung and breast cancer cells [52]. However, it is worth noting that the RNA contained in milk exosomes can directly or indirectly affect the growth of cancer cells, and this effect may be either inhibition or promotion [53]. Therefore, when milk exosomes are used as drug delivery vehicles, the influence of their natural sources on the human body should be fully considered [54].

Bacterial-derived extracellular vesicles (EVs) are often candidates for mucosal vaccines to induce immune protection against specific pathogens [55]. In addition, bacterial-derived EVs in urine can be used as novel biomarkers for the noninvasive diagnosis of gastric cancer [56]. Similarly, EVs from gut microbiota are associated with inflammatory bowel disease and colorectal cancer (CRC) [57]. Furthermore, EVs of the gastrointestinal microbiota play a role in brain dysfunction and neurodevelopment by modulating immune responses [58]. However, there have few studies on bacterial-derived EVs in the field of tumor therapy.

5. Isolation of Exosomes

To effectively and safely delivery drugs, exosomes must be isolated using efficient and reproducible isolation procedures. Exosome isolation techniques have been created based on density, size, and immunogenicity properties. Exosome purity, quantity, and physicochemical properties vary depending on isolation conditions [59] (Table 1, Ref. [60–81]).

5.1 Ultracentrifugation

Ultracentrifugation is the most popular and widely accepted gold standard for isolating exosomes [60]. It includes two types: differential ultracentrifugation and density gradient ultracentrifugation. Differential ultracentrifugation separates exosomes from other components by using different centrifugal forces and time cycles during the centrifugation process, mainly because different molecules have different particle sizes [63]. In density gradient ultracentrifugation, a continuous or discontinuous density gradient in a centrifuge tube is established with a specific medium, followed by the addition of a sample to the top of the medium to distribute different molecules in different fractions through gravity and centrifugal force fields, thereby separating exosomes and other components [63]. Ultracentrifugation requires fewer reagents and less operator expertise. Still, it may induce aggregates comprising various EVs and may be contaminated by lipoproteins with similar density [61,62].

5.2 Ultrafiltration

Ultrafiltration uses membranes with precise pore sizes to separate particles in a predetermined size range. Ultrafil-

Table 1. Comparisons of exosome isolation techniques.

Isolation technique	Principle	Advantages	Disadvantages	References
Ultracentrifugation	Size, density	The gold standard, simple operation, and low requirement for professional knowledge	Low purity, time-consuming, expensive equipment, and easy formation of aggregates	[60–63]
Ultrafiltration	Size, molecular weight	Short time-consuming and no special equipment required	Decreased yield due to membrane adsorption, contamination caused by deformation and rupture of large vesicles, and clogging of pores	[61,63,64]
Size Exclusion Chromatography (SEC)	Size	Small changes in exosome properties and efficient elimination of protein contamination	Time-consuming, lipoprotein contamination, protein aggregation, and equipment required	[65–67]
Flow Field-Flow Fractionation (F4)	Size	Low sample size requirements, fast and highly reproducible	High requirements for operator expertise	[63,68,69]
Hydrostatic Filtration Dialysis (HFD)	Size	Achieve efficient pretreatment and concentration of samples with good reproducibility	Blockage of nanomembranes	[63,70–72]
Polymer-Based Precipitation	Solubility, surface charge	No need for special equipment; fast and also suitable for large-scale isolation	Free protein contamination and low yield	[61,73–76]
Immunoaffinity Capture-Based Technology	Immunoaffinity	High purity and rapid isolation of specific populations	Low yield and limitation of exosome labeling	[77–79]
Microfluidics-Based Exosome Isolation	Immunoaffinity, size, density, etc.	Rapid, low-cost, less reagent and sample volume, potentially isolating source-specific exosomes	Small sample volume and lack of method validation and standardized testing	[63,80,81]

tration alone or combined with ultracentrifugation and gel filtration chromatography is effective for exosome isolation [82]. Compared to traditional ultracentrifugation, ultrafiltration improves the purity and integrity of the separated vesicles while taking less time. The adsorption of exosomes on the filter membrane can result in decreased yield. Orifice holes may lead to deformation and rupture of large vesicles or platelets, which may skew the results [64,65,77].

5.3 Size Exclusion Chromatography

SEC can be employed to isolate exosomes depending on the porous stationary phases that classify macromolecules and particles according to their size. Components with smaller hydrodynamic radii can enter these pores, resulting in extended retention durations, whereas exosomes and other substances with large hydrodynamic radii are excluded from the pores, thereby having a shorter retention time [63]. To ensure that the resolution in SEC meets the experimental requirements, the analysis is usually carried out at relatively low flow rates by using a rather long column (or multiple columns in series), making the separation process time-consuming. To address this shortcoming, several companies have developed commercial kits that enable higher separation efficiency and faster analysis [74].

5.4 Flow Field-Flow Fractionation

F4 is a hydrodynamic-based technique to separate exosomes by diameter differences. Fractionation occurs in a rectangular flow field with migratory flow moving along the axis of the flow field, whereas sample retention depends on the rate of the secondary flow (cross flow in F4). The lateral flow acts as an external field to induce movement of the sample towards the flow field walls. Due to diffusion, the sample is distributed at different locations on the flow field walls, which can be separated according to the size of the sample particles. The distribution layer formed by the smaller particles has a higher average height above the stacking wall than the larger particles and is eluted earlier, enabling the isolation of exosomes [68]. Combined with an assay system, this method allows the rapid isolation and characterization of exosomes [63].

5.5 Hydrostatic Filtration Dialysis

HFD is mainly used to isolate urinary extracellular vesicles by diffusion of solutes across a cellulose ester dialysis membrane with a defined molecular weight cut-off. The solvent will also pass through the dialysis membrane under hydrostatic pressure, thereby achieving filtration-concentration dialysis [71]. The advantage of HFD is that it can efficiently pretreat and concentrate samples and does not involve the operation of highly specialized equipment, making it a convenient method. HFD is significantly better for a wide range of sample volumes than differential centrifugation [70].

5.6 Polymer-Based Precipitation

Polymer-based precipitation utilizes the interaction of polymers such as polyethylene glycol with water molecules or the use of salt solutions such as sodium acetate to neutralize the negative charge of phosphatidylserine on the surface of exosomes, thereby reducing the solubility and dispersibility of exosomes and forming aggregates. Finally, the exosome particles can be harvested by centrifugation [61,65,73]. The most commonly used commercial polymer precipitation product for exosome isolation is the ExoQuick proprietary polymer from System Biosciences (Santa Clara, CA, USA) [83]. Compared with other methods, the exosome RNA isolated by the ExoQuick precipitation method has the largest amount and the highest purity [84]. However, exosomes isolated by this method are susceptible to contamination by free proteins such as viral particles, immunoglobulins, and lipoproteins [74]. Higher purity EVs generally have a higher ratio of particles (mainly vesicle components) to proteins (vesicular content proteins and other contaminating proteins), so this ratio can be directly used to reflect the purity of EV samples [85].

5.7 Immunoaffinity Capture-Based Technology

There are a large number of proteins and receptors on the exosome membrane. Immunoaffinity capture uses the specific binding of receptors and their ligands or the immunoaffinity between these proteins and their specific antibodies to separate exosomes [63]. Surface receptors such as CD9, CD63, and CD81 are considered biomarkers of exosomes and are often used to extract exosomes by the immunoaffinity capture method [86]. The main techniques developed based on this principle are the enzyme-linked immunosorbent assay in microtiter plate format and affinity capture based on immunomagnetic beads [60]. Given the specificity of the interaction between the antigens on the exosome membrane and the corresponding antibodies, theoretically, specific antibodies for tumor-associated antigens can separate tumor-derived exosomes from exosomes of nontumor origin. The problem is that these antigens may be overexpressed in tumor cells, but their concomitant expression on normal cells can lead to immune capture of exosomes in the patient's plasma, making it impossible to guarantee that all isolated exosomes are derived from tumor cells [87]. Nonetheless, as a rare exception, epitopes of melanoma-associated antigens are expressed only on melanoma cells and not on normal cells. Researchers have used immunoaffinity capture technology to separate melanoma-derived exosomes from other normal cell-derived exosomes in patient plasma [87]. In addition, isolation using immunoaffinity capture technology was shown to be better than centrifugation and density-based methods for the isolation of human colon cancer cell line-derived exosomes [78]. However, selectivity can also result in concomitantly lower yields compared to procedures that rely on isolation by physical properties, as some markers may not be presented or recognized on all vesicles within a given class [77].

5.8 Microfluidic-Based Exosome Isolation

Microfluidic-based exosome isolation is a high-throughput method that uses a microfluidic device to isolate exosomes based on several characteristics including size, density, and immunoaffinity. In addition, there are many novel isolation methods in the microfluidic platform such as nanowire trapping, acoustics, lateral displacement, and viscoelastic flow [59,88]. Compared with other existing exosome isolation methods, microfluidic methods are faster, less expensive, require fewer samples and reagents, can potentially isolate exosomes of specific cell origin, and retain most microfluidically isolated exosomes in their native form [80]. Davies *et al*. [89] isolated exosomes from whole blood via a microfluidic platform using pressure- or electrophoresis-driven, *in situ* photopatterned porous polymer monoliths as filter membranes. Microfluidic devices offer several advantages for utilizing exosomes as diagnostic tools, such as low cost, reliability, real-time diagnosis, and the ability to process microvolume liquid samples such as saliva, breast milk, blood, and urine. However, its sample capacity is too small, which is a significant disadvantage [81].

6. Drug Loading on Exosomes

The drug-loading methods in exosomes can be divided into presecretory and postsecretory drug loading according to whether the drugs are directly loaded on exosomes [90] (Table 2, Ref. [11,61,65,90–103]). As the name suggests, presecretory drug loading refers to drug loading during exosome biogenesis in cells before vesicle isolation. Drugs are loaded into exosomes using the cell's endogenous machinery by being added to the culture medium [65]. This creates the biggest problem, which is the inability to control the amount of drug encapsulated in the exosomes [61]. This mechanism includes co-incubation of the drug with the cells and transfection. Correspondingly, post secretion drug loading, which loads drugs into the isolated exosomes directly, obviously has higher efficiency, and the encapsulation efficiency and loading capacity of exosomes are easier to control [91]. Electroporation, sonication, freeze-thaw cycles, extrusion, co-incubation of drugs with exosomes, surfactant treatment, and dialysis all belong to this mechanism [61].

6.1 Presecretory Drug Loading

6.1.1 Co-Incubation (Drug and Cells)

In the co-incubation method, the drug is co-incubated with the cells, so the drug enters the cell and is then secreted in the exosome cargo. It is characterized by a simple operation but low drug-loading efficiency and the inability to control the production and release process of drug-loaded exosomes [11]. Some drugs may be toxic, affect-

Table 2. Comparisons of exosome drug-loading technology.

Loading mechanism	Loading technique	Advantages	Disadvantages	References
Presecretory drug loading	Co-incubation (Drug and Cells)	Easy operation	Low loading efficiency, difficulty to control, and the effect of drug toxicity	[11,61]
	Transfection	Overexpression of specific molecules	Low loading efficiency, the toxicity of transfection reagents, and micelles used for transfections that are not easily separated from exosomes	[61,92]
Postsecretory drug loading	Electroporation	The gold standard	Low loading efficiency, aggregation of exosomes, and reduced activity caused by the destruction of the protein drug structure	[90,91,93]
	Sonication	High loading efficiency, not easy to form aggregates, and retention of membrane surface adhesion proteins	Altered immune privilege status and disruption of exosome integrity	[94–97]
	Freeze-thaw Cycle	Easy operation	Low loading efficiency, exosome aggregation, and inactivation of protein drugs	[61,95]
	Extrusion	High loading efficiency, the most efficient water-soluble cargo loading technology, and unaffected exosome internalization properties	Altered immune privilege status and disruption of exosome integrity	[61,98]
	Co-incubation (Drug and Exosomes)	Easy operation	Low loading efficiency and susceptibility to drug hydrophobicity	[65,99]
	Surfactant Treatment	High loading efficiency and little impact on the physicochemical properties of exosomes	Hemolytic activity of saponins, requiring additional purification steps	[100,101]
	Dialysis	High loading efficiency	Degradation of protein or peptide drugs and changes in the size distribution of exosomes	[101–103]

ing the state of cells and secretion of exosomes [61]. Wang *et al*. [104] co-incubated cur with macrophages to obtain Exo-cur, which increased cur's solubility and stability and resulted in sustained drug release. More importantly, Exo-cur can fuse with the microvascular endothelial cell membrane in the brain through the specific binding of lymphocyte function-associated antigen 1 (LFA-1) and intracellular adhesion molecule-1 (ICAM-1) and promote cur to cross the BBB into the brain, making it potentially able for Alzheimer's disease-targeted therapy.

6.1.2 Transfection

Transfection is the most common way to package peptides, proteins, nucleic acids, and other active molecules into exosomes. The target gene is introduced into cells through a transfection process to express the cargo of interest, which is then encapsulated into exosomes by the cell's machinery. Of course, chemical treatment can also introduce nucleic acids into exosomes, a postsecretory drug-loading method [102]. Although transfection can ensure that the drug is loaded into exosomes before being secreted outside the cell, the loading efficiency is very low due to the uncontrollable drug-loading process. Direct chemical transfection always causes damage or contamination of exosomes [61]. HEK293 cells are transfected with plasmids and miRNAs, and purified in the culture supernatant to obtain miRNA-loaded exosomes modified with epidermal growth factor or GE11 peptides, which can target miRNA delivery to epidermal growth factor receptor-expressing cancer tissues [105]. However, chemical transfection is unsuitable for exosomes as gene delivery vehicles, because chemically transfected micelles can attach to exosomes nonspecifically, making the two inseparable [92].

6.2 Postsecretory Drug Loading

6.2.1 Electroporation

Electroporation uses short-term high-voltage pulses to form pores in the exosome membrane so that drugs can enter the interior of the exosome through these pores. This method is commonly used to enhance the uptake of siRNA, iron oxide nanoparticles, and small molecule drugs [106]. However, electroporation of siRNA into exosomes causes extensive precipitation of siRNA, which leads to the easily overestimated encapsulation efficiency of electropora-

tion [107]. Membrane stabilizers such as trehalose prevent the aggregation of exosomes after electroporation to a certain extent [93]. It is important to note that electroporation is limited to certain RNAs, whereas miRNA, short hairpin RNA (shRNA), and mRNA cannot be loaded into exosomes using this method [108]. Studies have shown that, within a certain range, changes in capacitance and siRNA will not affect the efficiency of electroporation. By contrast, the concentration of exosomes will affect electroporation efficiency [92]. Another disadvantage of the electroporation technique is low loading efficiency due to the disruption of exosomal membrane integrity and low biological activity due to disruption of the protein structure [90]. Electroporation is widely used as a gold standard method [91]. For example, electroporation can encapsulate PTX into exosomes to overcome tumor multidrug resistance [94].

6.2.2 Sonication

During sonication, the exosomes purified from host cells and the drug of interest are mixed, and then homogenized using an ultrasonic probe. Using mechanical shear force disrupts the integrity of exosome membranes, allowing drugs to enter exosomes during membrane deformation [99]. After sonication, the high stiffness of the exosomal membrane is reduced, which enables the drug to be incorporated into the lipid bilayer, which improves the loading capacity [94]. In addition, the drug may be attached to the surface of exosomes, which may be the reason for the explosive release of the drug in the early stage and sustained release in the later stage [94]. Notably, drug-loaded exosomes prepared by sonication showed nonspherical morphologies with various shapes, and disruption of exosome integrity during sonication may lead to changes in its immune-privileged state [95]. This method has significant advantages. Sonication can increase the entry of various small nucleic acids into EVs, and the sonicated EVs still maintain their integrity and the ability to transport small RNA cargoes. Sonication also forms fewer aggregates than electroporation [96]. Furthermore, EV membranes retain specific adhesion proteins that are particularly important for their interaction with target cells after sonication [97]. It should be noted that both the hydrophobicity of the drug and the temperature during sonication affect the loading of the drug [97]. Both PTX and doxorubicin can be loaded into EVs by this method as a *de novo* drug delivery strategy for treating triple-negative breast cancer [97].

6.2.3 Freeze-Thaw Cycle

In the freeze-thaw cycle, the mixed solution containing exosomes and drugs is incubated at 37 °C, snap-frozen at –80 °C, and thawed at room temperature; this is repeated for at least three cycles [109]. This method is easy to perform but with low drug-loading efficiency. The drug-loaded exosome particles prepared are large and consist of dozens of smaller exosomes, which may be caused by aggregation [95]. Through freeze-thaw cycles, exosomes can be fused with liposome membranes to obtain engineered hybrid exosomes. The fusion of proteoliposomes and exosome membranes allows functional lipids and membrane proteins to be embedded in exosomes, thereby improving the interaction between exosomes and cells [110]. However, the disadvantage of repeated freezing and thawing is that the protein may be inactivated [61]. This method was used to load therapeutic protein peroxidase into exosomes for the treatment of Parkinson's disease (PD) [95].

6.2.4 Extrusion

Extrusion is a physical process. When the mixture of drugs and exosomes is extruded through a polycarbonate porous membrane of a certain pore size, the exosome membrane is damaged and ruptured, and the drugs in the mixture are uniformly loaded into the exosomes [109]. This method is highly efficient for loading and is considered the most efficient technique for loading water-soluble cargoes. Still, reorganization of the exosome membrane may alter its immune-privileged state, making it visible to immune cells [61,98]. The catalase-loaded exosomes prepared by extrusion are in a dominant position in evaluating loading efficiency and catalytic activity of the enzyme, and showed stronger neuroprotective effects [95]. In addition, exosomes retain their spherical structure after extrusion, and extrusion does not affect the internalization properties of exosomes [111].

6.2.5 Co-Incubation (Drugs and Exosomes)

During the co-incubation of drugs and exosomes, they are simply incubated at a specific temperature, and the drug concentration gradient inside and outside the exosome pushes the drug into the exosome by diffusion [99]. This method mainly depends on the hydrophobic contact and diffusion between the exosome membrane and the drug, so the hydrophobicity of the drug is one of the decisive factors for loading efficiency [61,99]. The method is simple to perform, but the disadvantage is that the loading capacity is low, and hydrophilic drugs cannot pass through the lipid layer of exosomes in the form of passive diffusion [65]. Blood exosomes were incubated with a dopamine-saturated solution for 24 hours at room temperature, and the isolated drug-loaded exosomes can significantly improve the disease phenotype in PD mouse models. The systemic toxicity of exosome drugs is substantially lower than that of free dopamine [112]. In addition, studies have shown that the anticancer drugs PTX and doxorubicin are introduced into exosomes by co-incubation, allowing them to cross the BBB to treat brain cancer [113].

6.2.6 Surfactant Treatment

Surfactants such as saponins can dissolve exosomal membrane molecules, thereby forming pores and leading to increased membrane permeability [102]. This approach

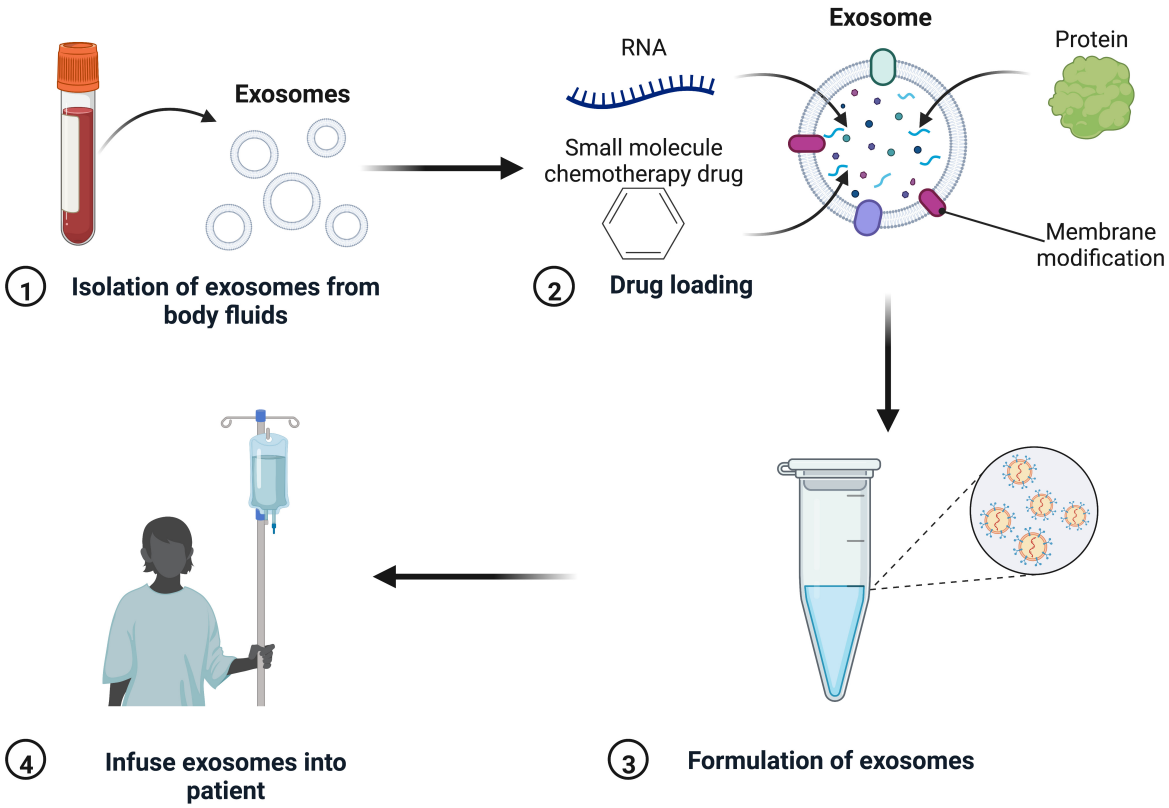

Fig. 3. Process flow of drug-loaded exosomes. Drugs are loaded into exosomes isolated from body fluids, and the exosome membrane can be modified. Exosomes need to be formulated before being applied to human therapy.

significantly enhances the drug-loading capacity in exosomes. In a study of porphyrin-loaded exosomes obtained by saponin treatment, it was found that saponin treatment did not change the particle size and zeta potential of drug-loaded exosomes, and the drug uptake capacity of exosomes was significantly improved after this treatment [101]. Similarly, after saponin treatment, the amount of catalase encapsulated in exosomes was increased considerably, and the enzymatic activity of catalase and prolonged and sustained release could be maintained [95]. Notably, saponins have hemolytic training, so their concentration should be strictly limited and they must be removed by an additional purification step [100].

6.2.7 Dialysis

In hypoosmotic dialysis, exosomes and drugs are transferred to a dialysis membrane or dialysis tube and placed in a near-neutral buffer, after which obtain drug-loaded exosomes are obtained by stirring dialysis [101]. Furthermore, the exosomes in the dialysis system can be pre-dehydrated in ethanol and then rehydrated in an acidic buffer to reduce the pH inside the exosomes and promote the formation of a pH gradient inside and outside the exosome membrane, thereby significantly improving the loading efficiency of exosomes [102]. However, dialysis may lead to the degradation of protein or peptide cargo [103]. In addition, the method may cause changes in the particle size of exosomes [101]. Therefore, before choosing this method, the class and properties of the drug should be considered, and suitable experimental conditions should be screened. This method is used to load moderately hydrophobic porphyrins; however, it has no substantial effect on porphyrin phototoxicity and shows poor cellular uptake [101].

7. Applications in Cancer Treatment

Based on their excellent properties, exosomes are widely used to deliver drugs such as small molecule chemotherapeutics, therapeutic nucleic acids, and proteins (Fig. 3). They have made meaningful progress in cancer therapy. The involvement of exosomes as drug delivery systems in cancer therapy for various drugs is summarized in Fig. 4.

7.1 Small Molecule Chemotherapy Drugs

Cur, a phytochemical with anticancer effects, can inhibit the transcription of the miR-21, thereby inducing PI3K/protein kinase B/phosphatase and tensin homolog (PTEN), nuclear factor kappa B (NF-κB), and programmed cell death protein 4 signaling pathways to affect apoptosis, migration, cell proliferation, drug resistance, and stemness, and ultimately exert inhibitory effects on cancer cells [114].

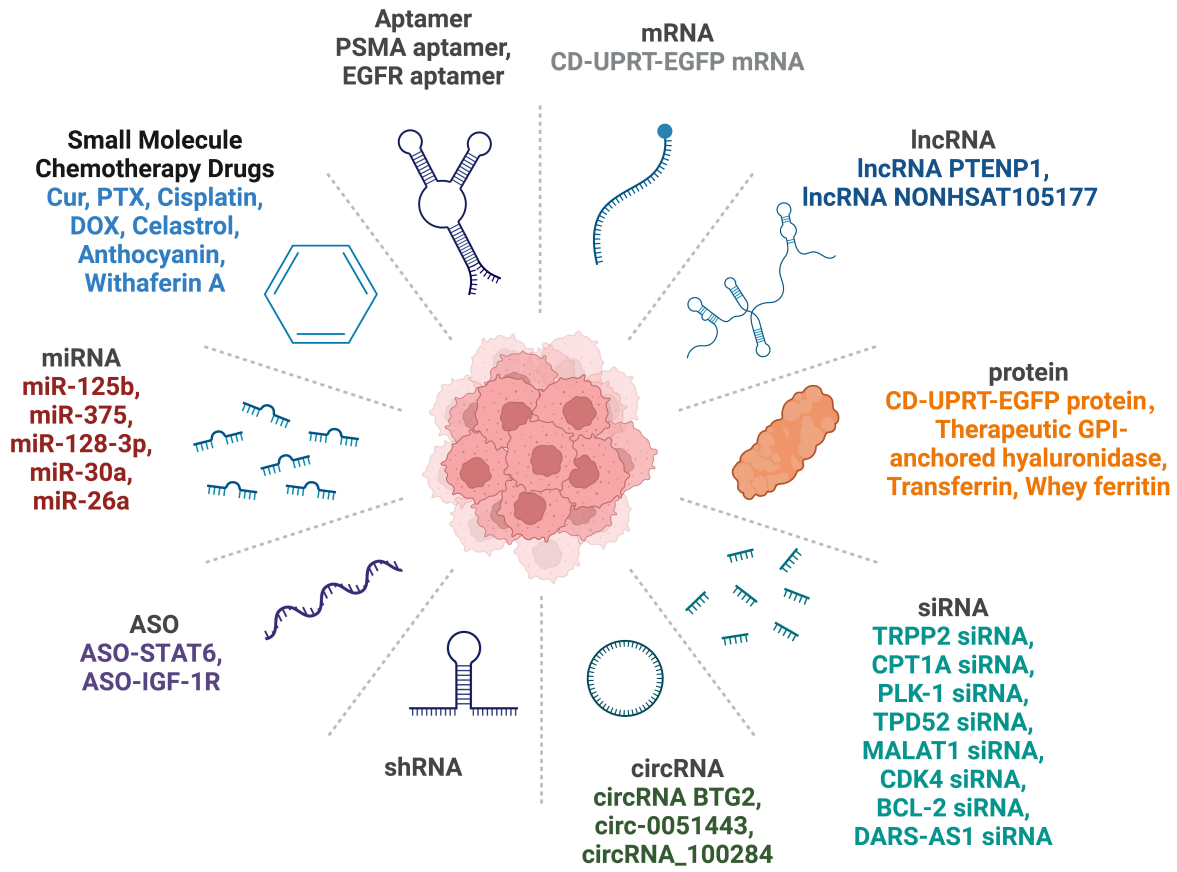

Fig. 4. Application of exosome-based cancer therapy. Different therapeutic agents are delivered by exosomes to treat cancer. We summarize the application of small molecule chemotherapeutics, miRNA, antisense oligonucleotides, shRNA, circRNA, siRNA, protein, lncRNA, mRNA, and aptamer in cancer therapy when exosomes are used as delivery vehicles.

However, the poor water solubility, poor stability, and low bioavailability of cur limit its use as a drug [115]. After loading cur into exosomes derived from milk and intestinal epithelial cells, they were both taken up by undifferentiated and differentiated intestinal Caco-2 cells, and the antiproliferative activity of cur was increased by 34% after incorporation into milk exosomes and by 26% in intestinal epithelial cell-derived exosomes [116]. In addition, the solubility of cur encapsulated in exosomes was significantly better than that in the free state. Exosomes can improve cur stability under extreme conditions such as high temperature, low or high pH, and enzyme treatment [115]. These properties make exosome-encapsulated cur a potentially effective oral anticancer drug [115].

Taxanes are a class of cytotoxic diterpenoids commonly used to treat solid malignancies [117]. The representative drug is PTX, which is used for the treatment of prostate, bladder, breast, ovarian, lung, and esophageal cancers [117]. Similar to cur, PTX is also poorly water-soluble, limiting its clinical application. The current solution is to bind PTX to albumin in a reversible non-covalent manner, increasing its solubility. This formulation is superior to isotoxic doses of standard PTX with a significantly lower incidence of toxicity [118]. Paradoxically, however, randomized controlled trials have shown that hematologic and non-hematologic toxicities, including peripheral neuropathy, are more prevalent with albumin-bound PTX than with conventional solvent-based PTX [119]. Another way to increase the water solubility of PTX is to dissolve it in a 50:50 mixture of Cremophor EL (CrEL) and ethanol. However, CrEL is not an inert carrier; it can exert a series of biological effects such as severe allergic reactions, hyperlipidemia, dyslipoproteinemia, red blood cell aggregation, and peripheral neuropathy [120]. Exosomes have also been used to load PTX [19]. Through exosomes secreted by M1 macrophages, PTX is delivered to tumor tissues. Studies have found that exosomes act as carriers to deliver higher amounts of PTX to tumor sites while activating the NF-κB pathway, which significantly enhances the antitumor effect [121]. Penetrating the BBB is a significant challenge in delivering anticancer drugs to brain tumors. By contrast, loading PTX into exosomes secreted by bEnd.3 murine immortalized brain endothelial cell line can significantly increase the cytotoxicity against U-87 MG glioblastoma cells, suggesting that exosomes with specific properties have the potential to deliver drugs through the BBB [113]. Similarly, Zhu *et al.* [122] demonstrated that c(RGDyk) peptide-modified human embryonic stem

cell-derived exosomes loaded with PTX have excellent glioblastoma-targeting ability.

Cisplatin is the first and most widely used metal-based chemotherapy drug. It is often used to treat solid tumors such as head and neck, stomach, lung, cervical, ovarian, bladder, and testicular cancers [123]. However, side effects and drug resistance limit its use in cancer therapy [123]. Zhou *et al*. [124] found that cisplatin could be delivered by milk-derived exosomes via clathrin-independent endocytosis and could avoid endosome capture and thus avoid drug resistance. Furthermore, exosomes secreted by M1 macrophages were shown to synergistically enhance the therapeutic effect of cisplatin in a mouse Lewis lung cancer model [125].

Doxorubicin (DOX) is a highly potent anticancer chemotherapeutic drug approved for the treatment of various human cancers [126]. However, its fatal cardiotoxicity limits its clinical application [127]. Encapsulation of DOX into liposomes can alter biodistribution, improve pharmacokinetics, and reduce toxicity [126]. On the downside, prolonged circulation of liposomes in the blood circulation may lead to adverse reactions in patients, including skin toxicity [128]. It has been reported that exosomes can serve as a suitable carrier of DOX. DOX was loaded into exosomes from the $A33^+$ LIM1215 CRC cell line, which could form complexes with A33 antibody-modified magnetic iron oxide nanoparticles (US) (A33Ab-US-Exo/Dox). The complexes can specifically target $A33^+$ colon cancer cells without causing significant cardiotoxicity [129]. The study by Wei *et al*. [130] also demonstrated that compared with free DOX, DOX loaded in exosomes had a much higher IC_{50} in the myocardial H9C2 cell line, indicating that exosomes as a carrier can significantly attenuate the cardiotoxicity of DOX. Similar to PTX, DOX can also penetrate the BBB via exosomes and thus be delivered to glioma cells (GMs). Notably, DOX-loaded autologous GM-derived exosomes inhibit the proliferation of parental GMs more than heterologous GMs, suggesting that autologous exosomes may be more favorable for drug targeting [131]. On the other hand, by labeling exosomes with targeting ligands, DOX can be delivered to specific sites. Covalently modified exosomes with the mucin 1 aptamer can be used to deliver DOX for the treatment of colon adenocarcinoma [132]. DOX is loaded into exosomes modified with the αv integrin ligand iRGD polypeptide. Both *in vitro* and *in vivo* experiments have shown that the iRGD-exosome drug can specifically target αv integrin$^+$ breast cancer cells [133].

7.2 Therapeutic Nucleic Acid

7.2.1 miRNA

miRNAs are a class of small RNA molecules of 19–22 nucleotides in length, which are present in all eukaryotic cells and can complementarily bind to the 3' untranslated region of mRNA, thereby negatively regulating the transcription of target genes [134]. miRNA-based cancer therapy has many advantages, such as the properties of broad regulation; that is, it can effectively silence target genes and simultaneously regulate other genes of interest to produce synergistic therapeutic effects. In addition, miRNAs have the properties of low toxicity and low immunogenicity [135]. However, miRNAs as drugs face many challenges such as poor blood stability and integrity, difficulty penetrating tumor tissues, and off-target effects [135]. Exosomes can be used as delivery vehicles to improve these problems.

In cancer patients, miR-375 expression is negatively correlated with the epithelial-mesenchymal transition (EMT). Using exosomes isolated from CRC cells as a carrier, Rezai *et al*. [136] delivered miR-375 mimics to HT-29 and SW480 CRC cell lines, significantly reducing their migration and invasion abilities. In addition, for oxaliplatin-resistant CRC cells, exosome-delivered miR-128-3p can restore the sensitivity of drug-resistant cells by suppressing the EMT-promoting oncogenes 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 and ubiquitin carboxyl-terminal hydrolase 2 [137]. Similarly, exosome-mediated miR-30a reverses the sensitivity of cisplatin-resistant oral squamous cell carcinoma cells to cisplatin by reducing Beclin1-mediated autophagy and inhibiting B-cell lymphoma 2-regulated apoptosis [138].

7.2.2 siRNA

siRNAs are approximately 21 nucleotides long and are derived from double-stranded RNAs that induce gene silencing by sequence-specific cleavage of entirely complementary mRNAs [139,140]. As natural drug delivery vehicles, exosomes can deliver siRNA to target tissues or cells *in vivo*, regulate gene expression, and inhibit tumor development [141]. It has the advantage of escaping from phagocytosis and low cytotoxicity and immunogenicity [141]. For example, when transient receptor potential polycystic 2 (TRPP2) siRNA was loaded into exosomes isolated from 293 cells, the formed complexes remained stable in the presence of nucleases and serum, and upon entry into FaDu cells (a hypopharyngeal carcinoma cell line), significantly inhibited TRPP2 expression, the EMT process, and migration and invasion [142]. On the other hand, tumor drug resistance can be overcome by siRNA delivered by exosomes. A study by Lin *et al*. [143] showed that carnitine palmitoyltransferase 1A (CPT1A) may be an essential target for reversing oxaliplatin resistance in colon cancer. The authors inhibited CPT1A by delivering CPT1A siRNA through iRGD exosomes, thereby inhibiting fatty acid oxidation and reversing colon cancer resistance to oxaliplatin [143]. Similarly, GRP78 siRNA delivered by bone marrow mesenchymal stem cell-derived exosomes into hepatocellular carcinoma cells can enhance the chemosensitivity of drug-resistant hepatocellular carcinoma to sorafenib to overcome the pharmacological resistance of sorafenib [144]. Additionally, to reduce endosomal trapping, Zheng

et al. [145] delivered siRNA via folic acid-modified exosomes with desirable results. Also, due to the upregulated expression of folate receptors on the membrane of many epithelial cancer cells, such folate-modified exosomes vigorously promote targeted delivery to tumors.

7.3 Other Therapeutic Compounds

Exosomes can also deliver other types of therapeutic drugs. For example, HEK-293T cells were used to stably express a fusion protein of uracil phosphoribosyltransferase and cytosine deaminase by transfection, and exosomes loaded with the fusion protein were isolated, which could convert 5-fluorocytosine prodrugs into active form to exert anticancer effects [146]. In addition to proteins and mRNAs, antisense oligonucleotides, long non-coding RNAs (lncRNAs), shRNAs, circular RNAs (circRNAs), and aptamers have also been introduced to exosome-mediated cancer therapy [147]. Zheng *et al*. [148] found that exosome-delivered lncRNA PTEN pseudogene 1 could regulate PTEN levels by competing with miR-17, thereby inhibiting bladder cancer progression. Similarly, circRNA BTG anti-proliferation factor 2-loaded exosomes from RBP-J-overexpressing macrophages were shown to inhibit the proliferation and invasion of glioma cells via the miR-25-3p/PTEN pathway [149].

7.4 Targeted Delivery of Exosomes

Specific cell-derived exosomes exhibit a natural tropism for the cancer microenvironment, which is critical for improving the targeting properties of nanomedicines [150]. Macrophage-derived exosomes, for example, can target cancer cells through their surface-expressed LFA-1 protein and ICAM-1 that is overexpressed in most cancer cells [97]. Similarly, exosomes from T cell lineages are taken up by myeloid cells, whereas mature DC-derived exosomes are efficiently internalized by activated T cells [151].

The artificial modification of exosome surface ligands can also promote the development of receptor-mediated tissue targeting [6,18]. Chimeric antigen receptor engineered T cells (CAR-T)-derived exosomes not only retain the targeting specificity of CAR-T cells but also can significantly reduce the side effects caused by CAR-based adoptive immunotherapy due to their cell-free nature and biological characteristics, thus replacing CAR-T cells as direct attackers for cancer therapy [152]. In addition, exosomes modified with polyethylene glycol and a somatostatin receptor 2 (SSTR2) monoclonal antibody enable targeted delivery of the histone deacetylase inhibitor romidepsin (FK228) to neuroendocrine carcinomas overexpressing SSTR2 cells, showing potency and low toxicity [153].

8. Challenges and Improvement Strategies

Exosomes have significant advantages as drug delivery vehicles, and based on their biological origin, they are less immunogenic. As aforementioned, the targeting properties of exosomes, either naturally or artificially modified, have significant implications for the targeted therapy of tumors. In addition, exosomes have excellent properties in prolonging blood circulation time and inhibiting tumors [16]. Nonetheless, in terms of clinical application, several challenges still hinder the application of exosome therapy.

8.1 Low Yield

The production of exosomes is limited, and is far from meeting the requirements for large-scale production. Methods such as ultracentrifugation or filtration are laboratory-scale-based methods for isolating exosomes and cannot meet the needs of clinical applications [16]. Measures to increase exosome production include bioreactors, three-dimensional cultures, and microfluidic devices [147]. Watson *et al*. [154] developed a hollow fiber bioreactor-based method for the large-scale production of high-purity bioactive EVs, which can increase the yield by 40 fold compared to traditional methods. However, the EV population obtained by this method is more diverse, which raises the requirements for subsequent purification [154]. The other method is a bioreactor culture system based on S/XF microcarriers, which increases the yield by 5-fold compared to conventional static systems, likely due to the aggregate formation and lower oxygen levels [155]. In addition, the study also confirmed that hypoxia could promote the secretion of exosomes by cancer cells, which may be mediated by hypoxia inducible factor alpha [156]. Likewise, stressors such as cytostatics increase exosome secretion from tumor cells [157]. It is worth noting that when increasing exosome secretion from cells by these physical or chemical factors, it should be carefully evaluated whether it will adversely affect exosome safety and therapeutic efficacy [16]. As another method to improve exosome yield, three-dimensional culture based on biocompatible collagen hydrogels can significantly improve the yield and collection efficiency of exosomes, and even modulate the biological function of exosomes [158]. The microfluidic co-flow system of the viscoelastic sample fluid and Newtonian sheath fluid reported by Liu *et al*. [159] can achieve 96% and 91% separation efficiency and recovery rate of exosomes below 200 nm, respectively. However, the current microfluidic platform is mainly suitable for small-volume sample separation. Expanding the device with a multichannel format may be one of the solutions for its future as a separation method for large-scale production [159].

8.2 Exosome Heterogeneity

Many studies have shown that exosomes from the same parental cell may have different molecular compositions [16]. In addition, the functions of a large part of the components are still unclear, such as some non-coding RNAs, lipids, and proteins. Incorporating these unknown biologically active molecules may bring certain risks [12]. Exosome cargoes such as miRNAs and lncRNAs, which

have been widely studied, may be involved in the process of inducing or promoting tumor formation, which is undoubtedly dangerous for patients [6]. This is of particular concern in exosomes secreted by tumor cells, although they have the advantage of potentially targeting specific tumors. In the clinical setting, MSCs-derived exosomes are not only safe and reliable but also have the benefit of high yield [160,161]. As drug delivery vehicles, the characterization of exosomes is crucial. Methods to characterize EVs with single particles such as transmission electron microscopy, flow cytometry, and nanoparticle tracking analysis have been reported, which have a driving role in understanding exosomes [162]. In addition, standardized quality management practices are critical to address the problems caused by exosome heterogeneity.

8.3 Short Half-Life in Vivo

Studies have shown that intravenously infused exosomal drugs are rapidly cleared by the reticuloendothelial system in the spleen and liver, resulting in reduced drug accumulation at the target site. This rapid clearance may pose toxicity concerns for chemotherapeutic drugs [17]. The half-life of exosomes *in vivo* can be prolonged by blocking receptors that mediate exosome uptake. Watson *et al*. [154] showed that blockade of the scavenger class A family of receptors significantly reduces EV uptake by macrophages/monocytes. Modification of the exosome membrane is another way to prolong its half-life *in vivo*. CD47 is one of the exosomal proteins, and in the case of its high expression, exosomes can escape the phagocytosis of circulating monocytes and macrophages. Their half-life is significantly prolonged [163]. This may be achieved through the interaction of CD47 with signal regulatory protein alpha on phagocytes [164]. In addition, surface modification of exosomes with polyethylene glycol significantly inhibits the nonspecific uptake of DCs [161].

8.4 Inefficient Loading

The low efficiency of current drug-loading methods largely limits their use, and the destruction of exosome integrity and drug stability is a common disadvantage of traditional methods. Some methods have been proposed to solve these problems to some extent. For example, exosome membrane protein CD9 is fused with RNA-binding protein human antigen R (HuR), and the resulting fusion protein recruits target RNA to exosomes through RNA-HuR recognition, markedly improving exosome nucleic acid-loading efficiency [165]. In addition, the chemical reagent-based method developed by Zhang *et al*. [166], which directly transfects miRNAs into exosomes, has proven convenient and efficient and can manipulate specific miRNAs in exosomes. On the other hand, many factors affect the loading efficiency of drugs such as the source of exosomes, the ratio of exosomes and drugs, and the properties of drugs [167]. These factors should be fully considered before the formulation design of exosomes, and the optimal parameters can be screened experimentally.

9. Conclusions

Exosomes have received much attention in the delivery of drugs or genes due to the benefits of both cell-based drug delivery and nanotechnology for effective delivery. This review focuses on using exosomes as drug delivery vehicles for treating tumors. Antitumor drugs in the form of proteins, nucleic acids, and small-molecule chemotherapeutics can all be effectively loaded into exosomes. Drugs that have been exosome-encapsulated may be used to create oral anticancer medications by increasing their water solubility and bioavailability. The BBB-penetrating properties of exosome-delivered medicines give them the significant potential for treating brain cancers. Exosome-based delivery of drugs can improve their stability and reduce immunogenicity, extending the use of nucleic acid products, particularly miRNA and siRNA, in cancer therapy.

The most critical steps in the generation of exosome-loaded antitumor drugs are the extraction of tumor cell-derived exosomes and the loading of drugs. We have systematically described the various methods for exosome isolation and drug loading. It is worth noting that each method has different application scopes, limiting their application in clinical. To meet the therapeutic demand for large-scale and highly purified exosomes in clinical, further studies are needed to optimize the isolation strategies.

The complexity of the contents of exosomes produced by cells and the fact that many of their roles are still poorly understood is another problem that prevents exosomes from being used in clinical settings. Notably, some miRNAs or lncRNAs enclosed in the exosome may be involved in the process of inducing and promoting tumor formation, which brings hidden risks for exosome-delivered drugs in treatment. One possible solution is using exosomes from non-tumor or even non-human sources, such as exosomes from bacteria, milk, and plants. These exosomes are characterized by low toxicity and easy availability. The exosomes derived from various sources can also potentially treat diseases other than cancer, such as inflammation, neurodegenerative diseases, *etc*. However, these need further study to verify the effects and potential risks of exosomes extracted from non-human sources in the human body.

Improving targeting and delivery efficiency should also be approved for the widespread clinical application of exosomal delivery strategy. Any cell can endocytose exosomes, which increases the off-target toxicity of exosome-based drugs. Isolation exosomes from the specific cells with naturally targeting or artificially modified exosomes with receptor-mediated tissue targeting are one of the current research hotspots in this field of exosome drug delivery. However, artificial modification under physical or chemical stimuli may change the physical properties of exosomes, such as changes in size and surface charge, which

might impact the therapeutic effect. Further studies on the mechanism of exosome generation and uptake, especially screening and identifying the characteristic markers that mediate the binding of exosomes to recipient cells, facilitate the development of artificially modified exosomes.

In conclusion, despite the obstacles that still need to be overcome, including the challenging of large-scale exosome isolation and drug loading technologies, potential risks brought by tumor-derived exosomes, the limited specific targeting of exosomes, the excellent properties of exosomes shown in basic research and preclinical trials strongly suggest their great potential as drug delivery vehicles in cancer therapy.

Author Contributions

YZ and NX wrote the manuscript. JL and WG revised the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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