**EXOSOMES - NATURALLY OCCURING DRUG DELIVERY VEHICLES: AN INSIGHT**

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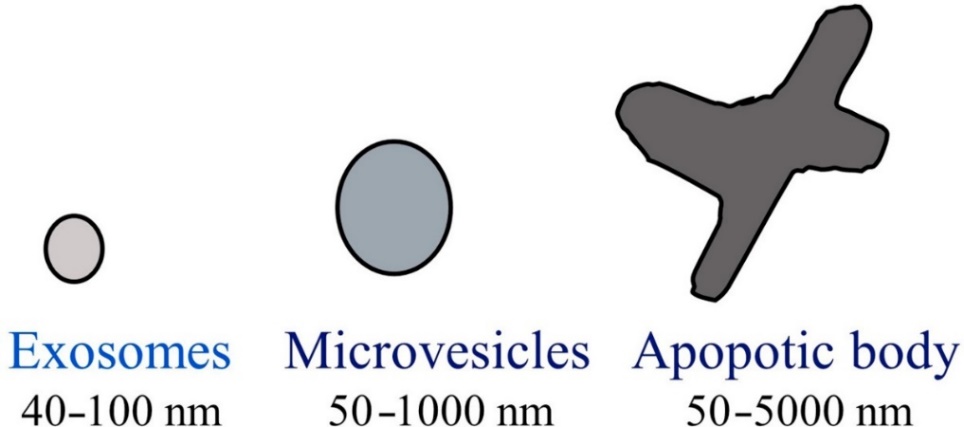
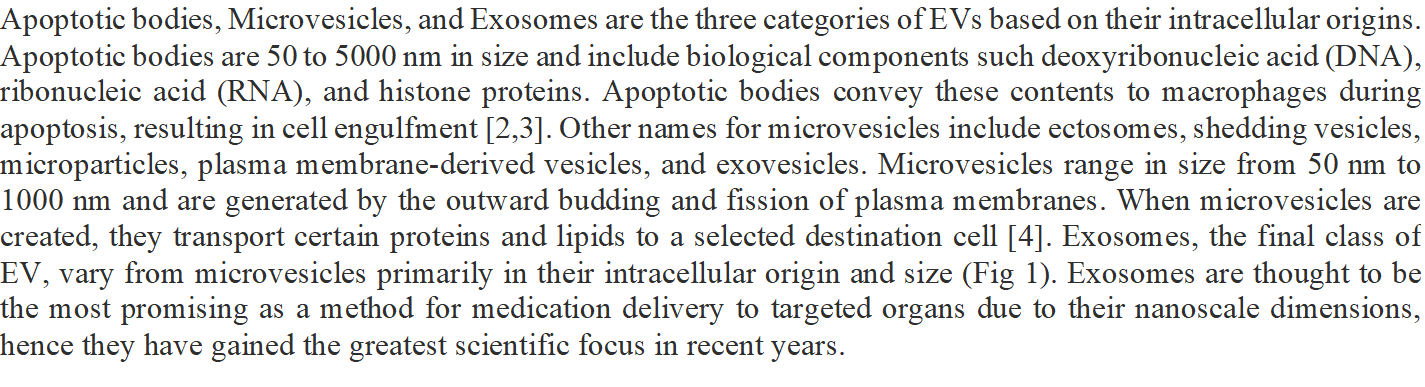
**ABSTRACT**

Exosomes are small intracellular membrane-based vesicles with a diameter of 40-100 nm that are secreted into the extracellular milieu by many cell types and are involved in a variety of physiologic and pathological processes. Exosomes offer significant advantages over other nanoparticulate drug delivery technologies such as liposomes and polymeric nanoparticles; exosomes are non-immunogenic in nature due to their similar composition to the body's own cells. Exosomes have a high potential for use as drug delivery vehicles because of their natural material transportation properties, intrinsic long-term circulatory capability, and excellent biocompatibility, making them suitable for delivering a wide range of chemicals, proteins, nucleic acids, and gene therapeutic agents. However, significant concerns and challenges remain, such as producing exosomes on a large scale for clinical application, determining which cell type to employ for exosome derivation, and determining in vivo exosome potency and toxicology. Exosomal therapeutics must be clinically translated by improving our understanding of exosome biology and function and developing nanotechnologies to precisely separate well-characterized clinical-grade exosomes and load them with a variety of therapeutic payloads.

**Key Words:** Drug Delivery Vehicle, Therapeutic agent, Nano technology, Biocompatible, Clinical application

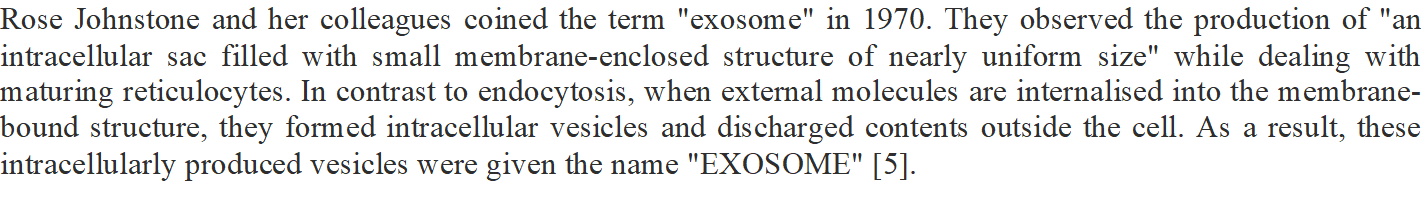
**INTRODUCTION**

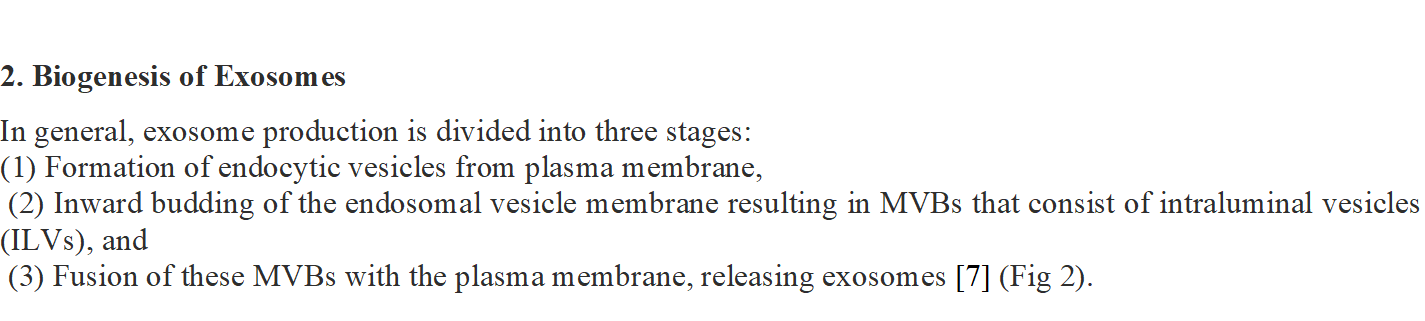
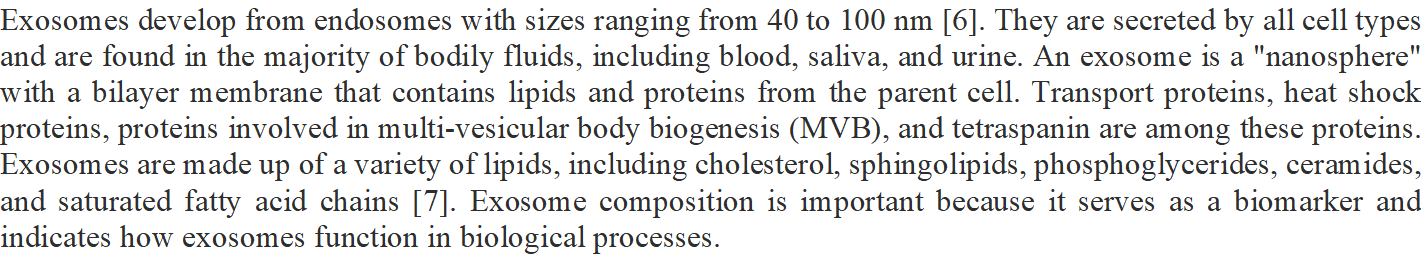
Chemical messengers are the primary means through which cells communicate with one another. Extracellular vesicles (EVs) are the most common type of these. Because of their unique structure, EVs can be modified to contain specific proteins, genetic lipids, and genetic materials such as messenger RNA (mRNA), microRNA (mRNA), and other small non-coding RNAs, and genomic DNA (gDNA) from their progenitor cell.[1]

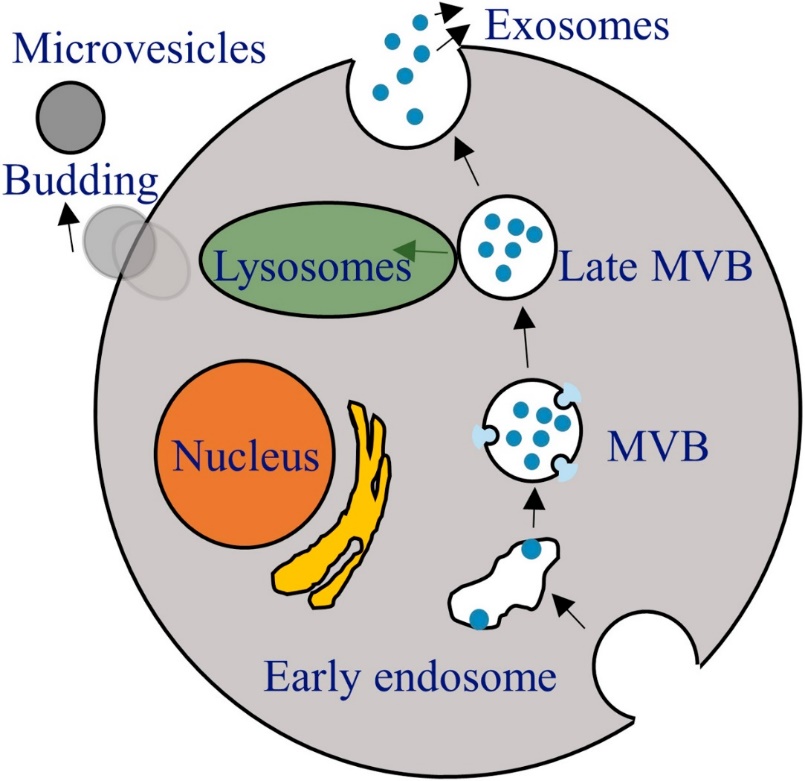


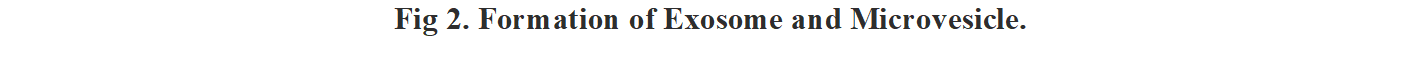
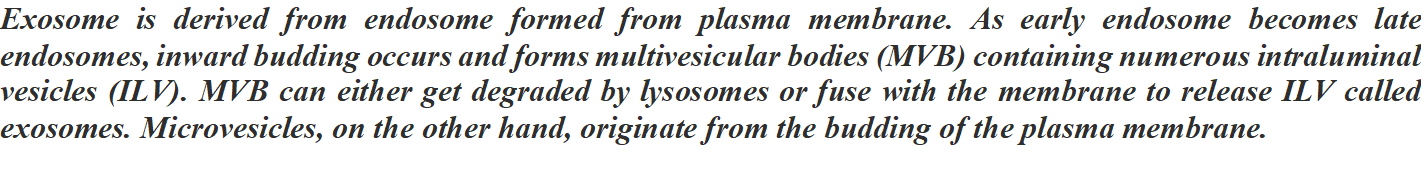
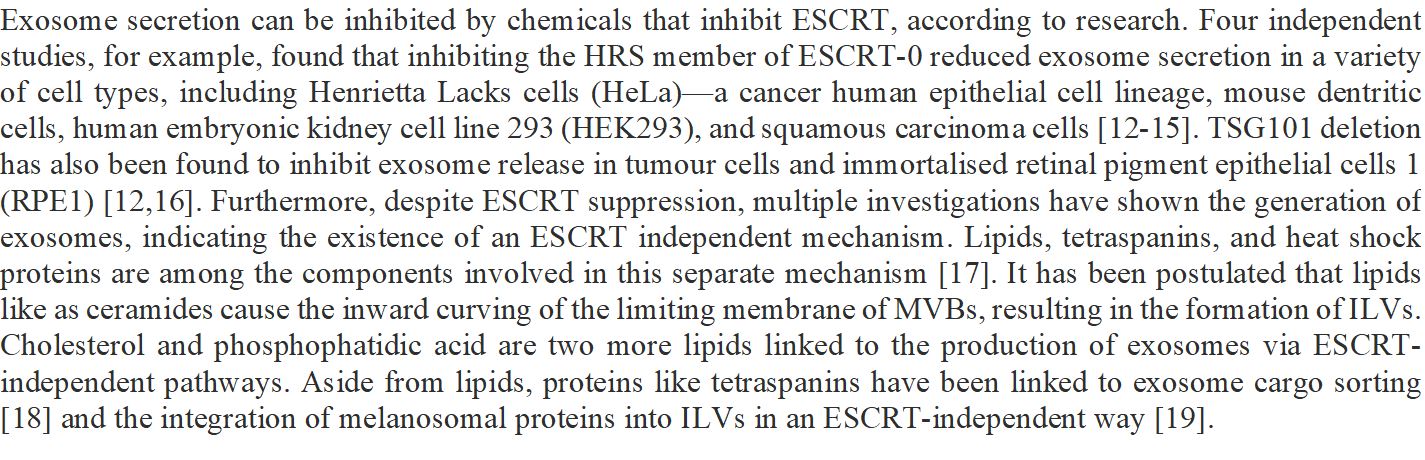
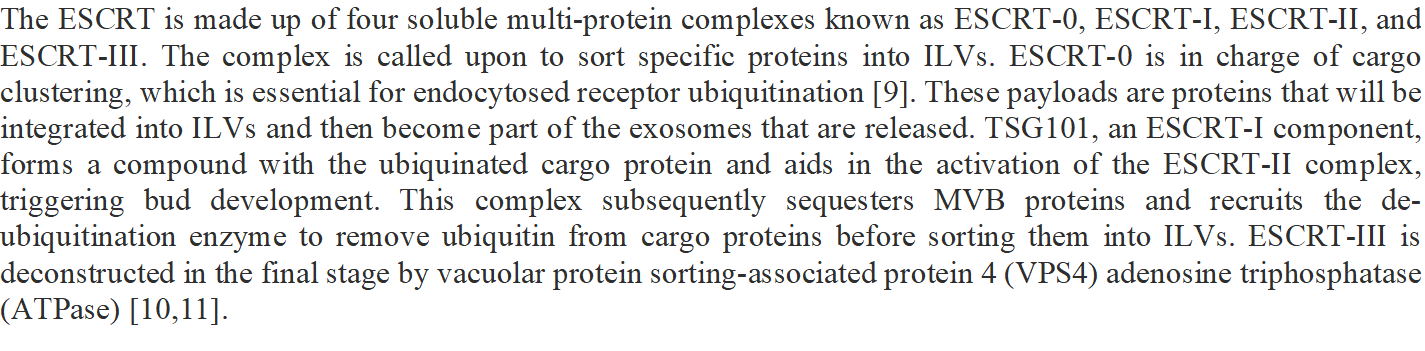
**Fig 1. Types of Microvesicles**

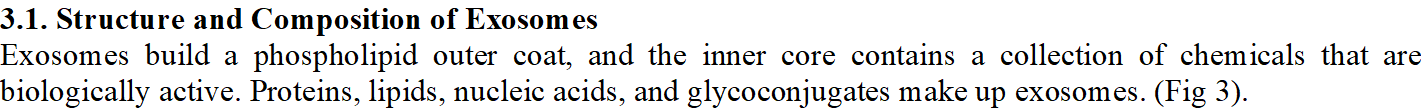
1. **Discovery of Exosomes**

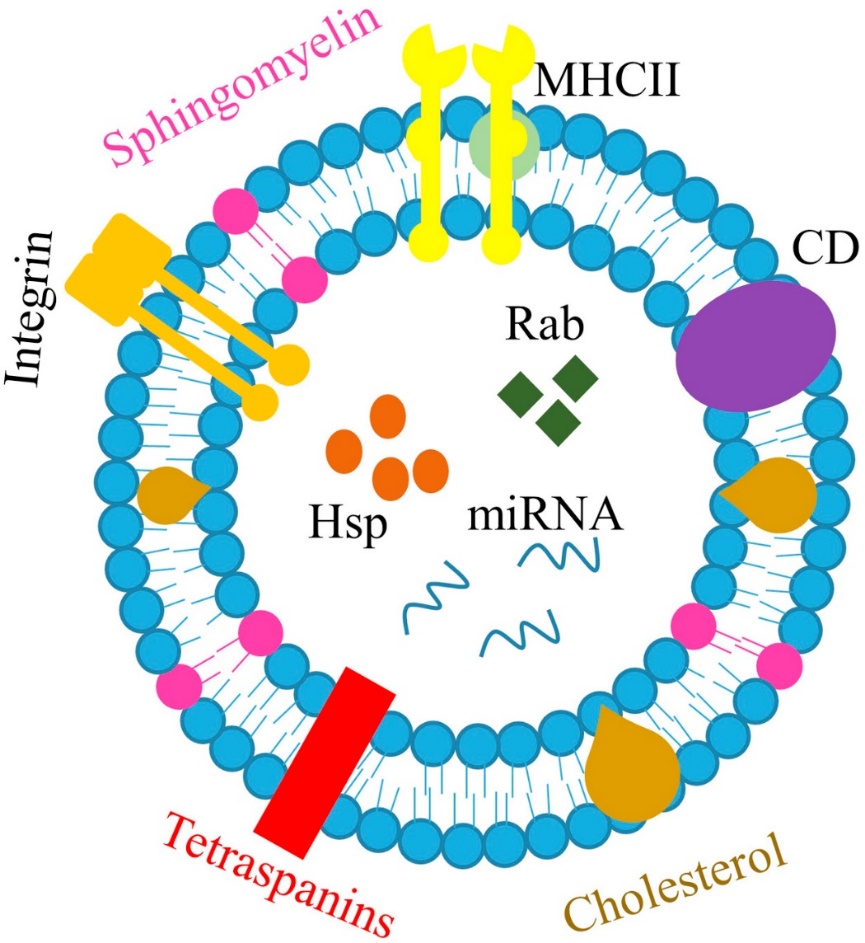


****Early endosomes are produced from plasma membrane endocytic vesicles and mature into late endosomes. Late endosomes create lumen vesicles by budding inward. MVBs are late endosome ILV accumulations. Two pathways produce MVBs. ESCRT-dependent and ESCRT-dependent pathways exist. MVBs can merge with lysosomes to destroy them or with the plasma membrane to release ILVs as exosomes [8].

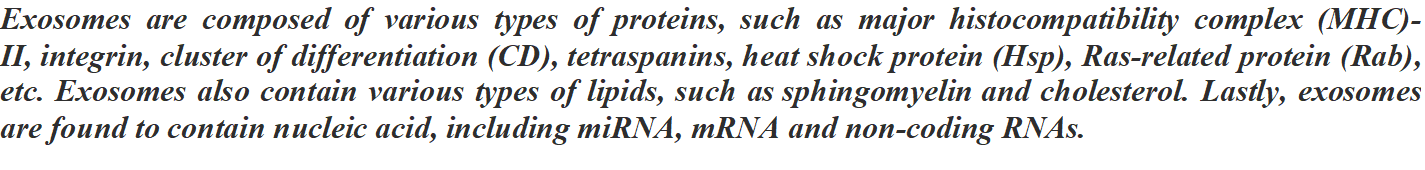


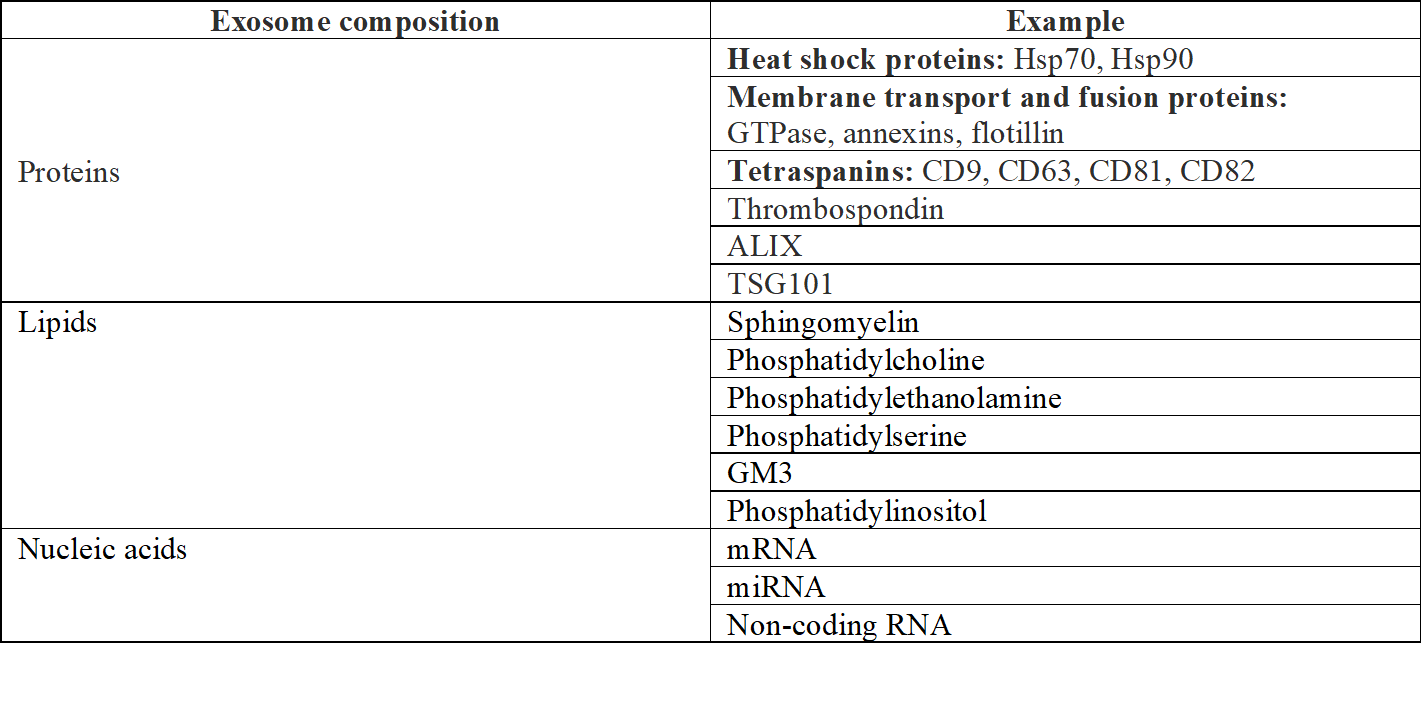
********3. Fundamentals of the Exosomes**

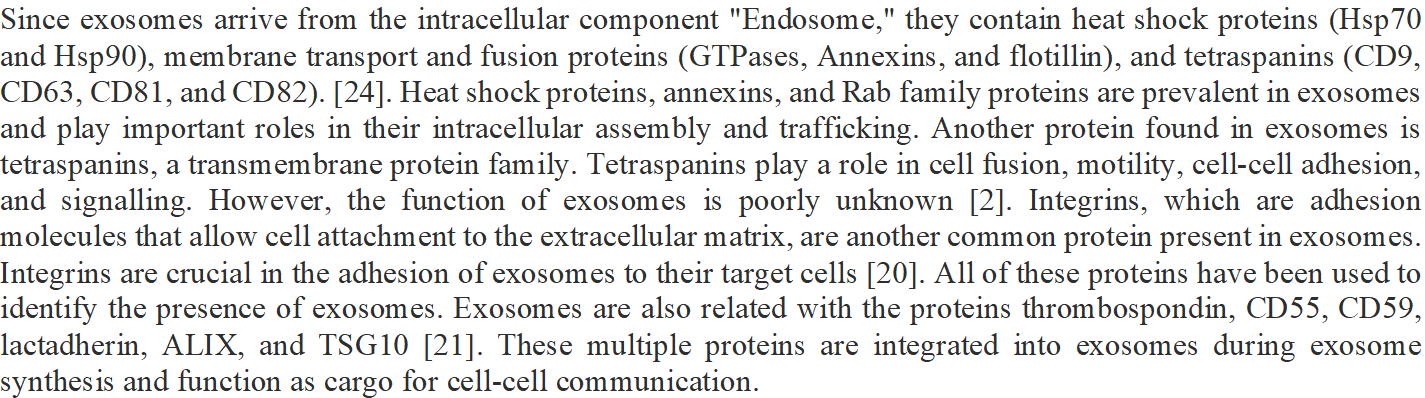
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**Fig 3. Exosome composition**

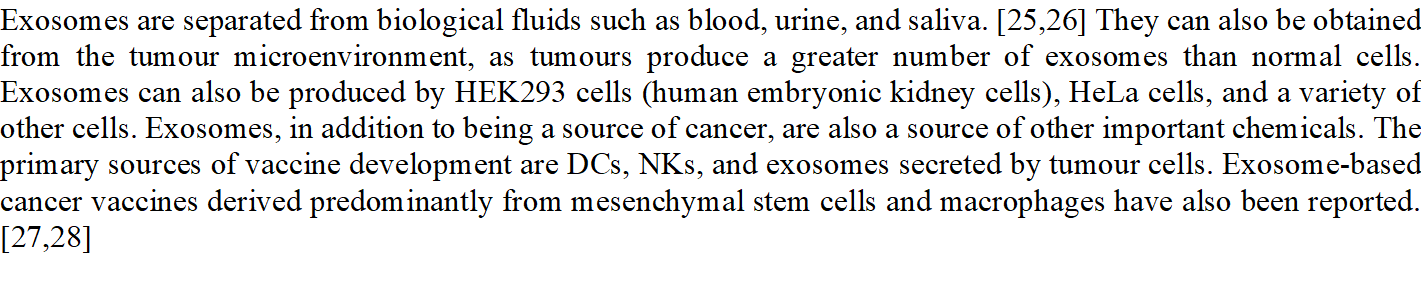
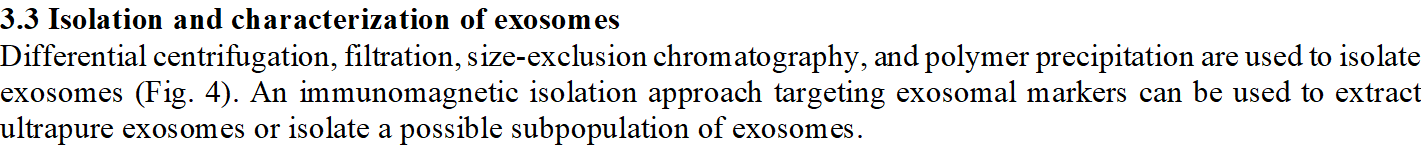
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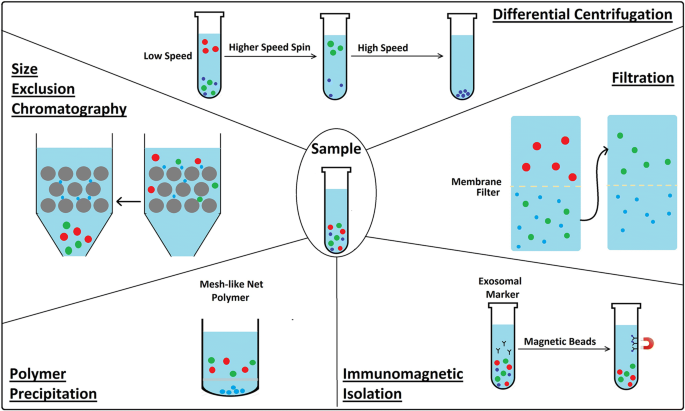
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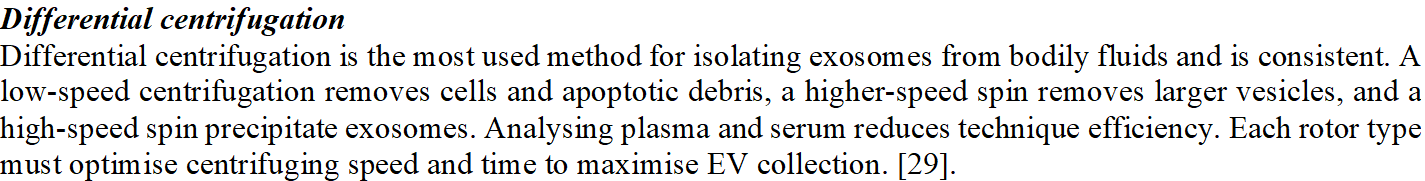


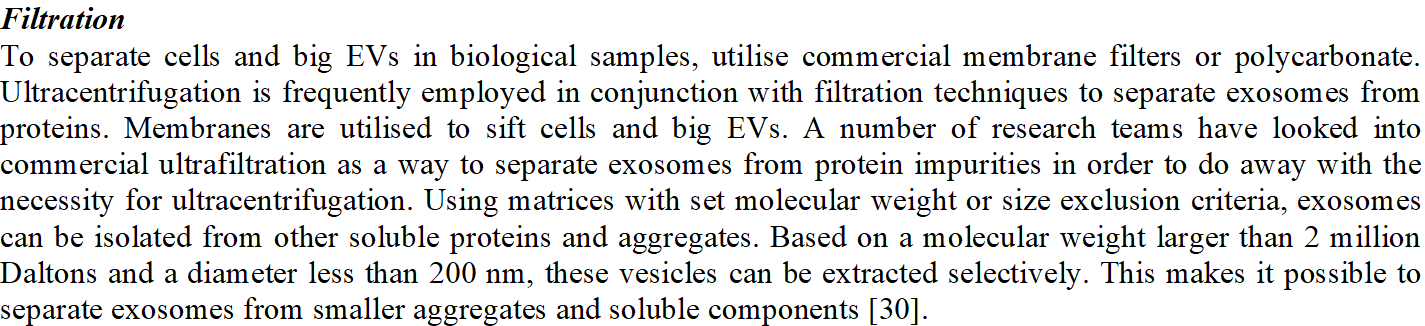
Exosomes contain lipids and proteins. Exosome lipid bilayers are mostly composed of lipids found in cell plasma membranes, such as sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidyl serine, mono sialo tetra hexosyl ganglioside (GM3), and phosphatidylinositol [22]. Sphingo-myeline and GM3 are responsible for exosome rigidity [23], whereas phosphatidylserine is expressed on the plasma membrane of exosomes via several phospholipid transporter enzymes. It is crucial in binding the outside proteins, allowing the exosome to communicate and fuse with the plasma membrane [24]. Cholesterol, ceramide, phosphoglycerides, and saturated fatty acids form exosomes. Exosomes include miRNA, mRNA, and non-coding RNAs.

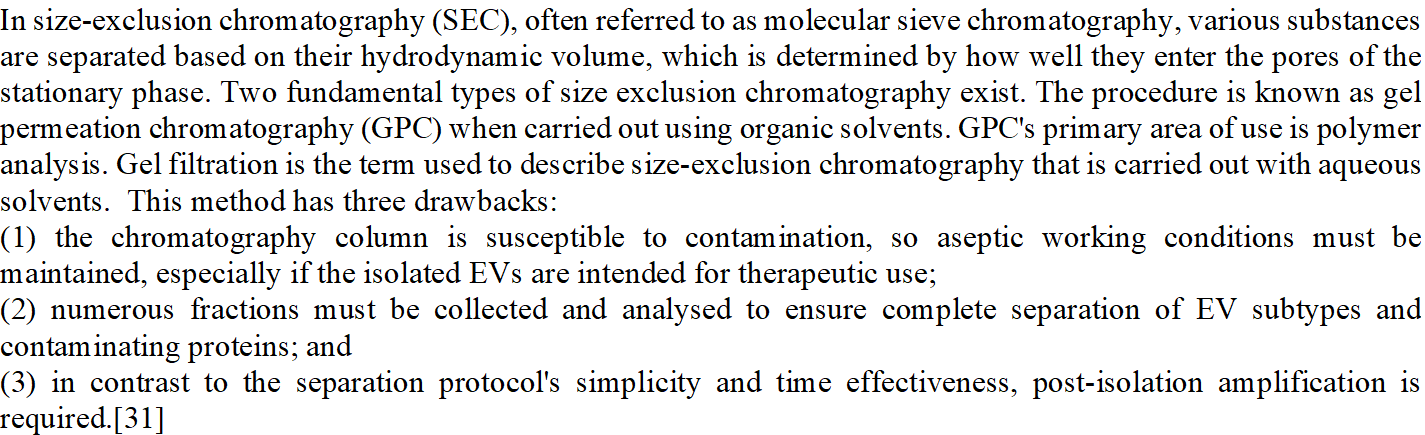
**3.2. Exosome Sources**

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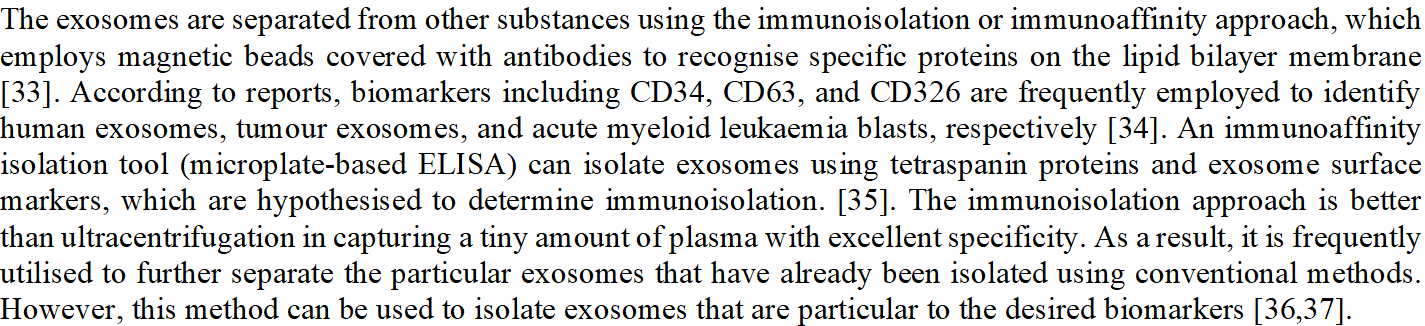
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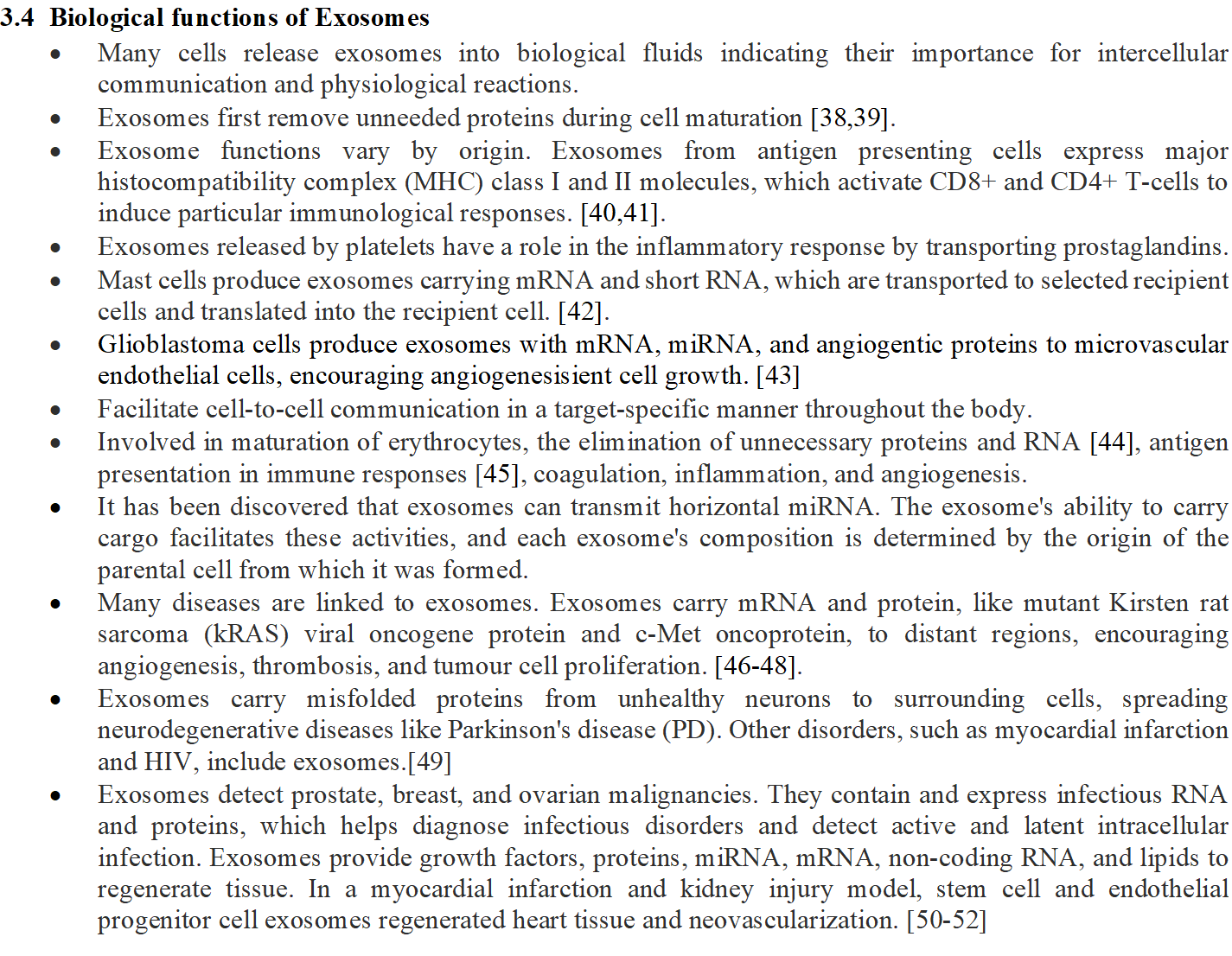
***Polymer precipitation***

Polymers are isolated and purified via a process called polymer precipitation. The foundation of polymeric precipitation techniques is the development of a mesh-like net into which EVs with sizes ranging from 60 to 180 nm are included. These techniques can be used on body fluids or on culture media. Polymeric precipitation techniques may be particularly advantageous for finding biomarkers in vesicles made from tiny biological samples [32].

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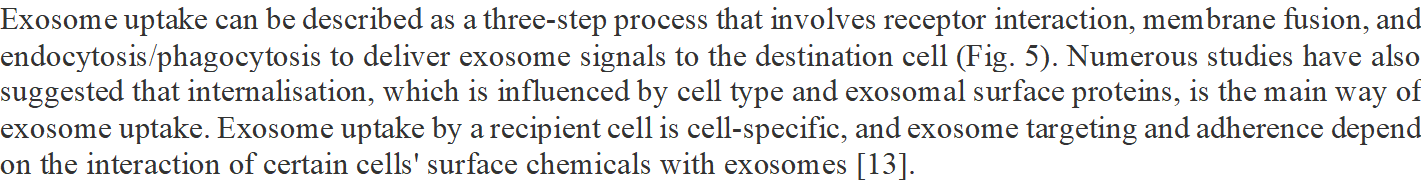
****The separation of exosomes has recently been made possible via microfluidics-based chip isolation techniques. These techniques are based on the distinction between the exosomes' physical and biochemical characteristics, including their size, density, and immunoaffinity. The three purification and isolation techniques utilising microfluidics-based chip isolation techniques are immunoaffinity for exosome trapping techniques, sieving techniques, and exosomes being adsorbed into the porous structure techniques [79]. All three methods necessitate sample preparation processes performed off-chip, such as reagent mixing and plasma extraction, which raises the level of processing complexity. This method specifically entraps exosomes that are between 40 and 100 nm in size, and the exosome specificity is strong, especially for the microfluidic-chip-based immunoaffinity capture approach. Exosomes can be separated from whole blood using the sieve method based on pressure or electrophoresis [80]. Some benefits of this technology are low cost, mobility, and quick sample processing. The capacity to effectively separate, purify, and inexpensively generate exosomes in large enough quantities, however, would restrict the use of this approach in clinical applications.

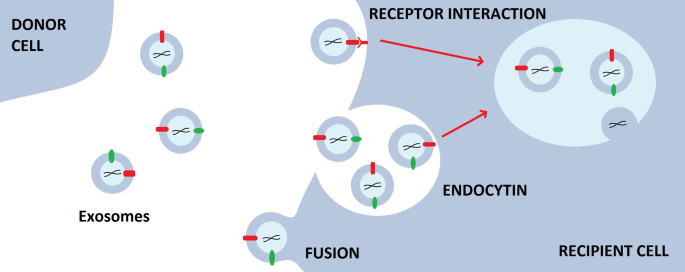
**3.5 Exosome Cellular Recognition**

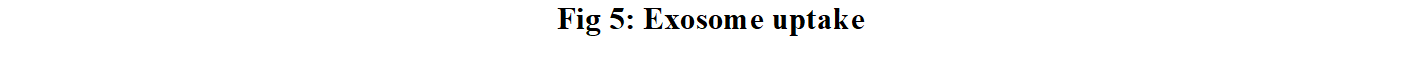
There are three different mechanisms for cells to recognise exosomes: free floating, adhesion, and antigen recognition. Similar to liposomes, exosome cellular recognition by free floating may be mediated by opsonization of exosomes during circulation. Leukocytes are drawn to the exosomes' location by chemokines. A wide variety of chemokines that are expressed by exosomes may draw T cells and other cell types.

Exosome adhesion is the first step in exosome-T cell interaction. Exosome adhesion requires the conformational change of integrins from low affinity to high affinity, which allows oligomerization and coupling with cytoskeletal components. [12].

**3.6 Exosome uptake**



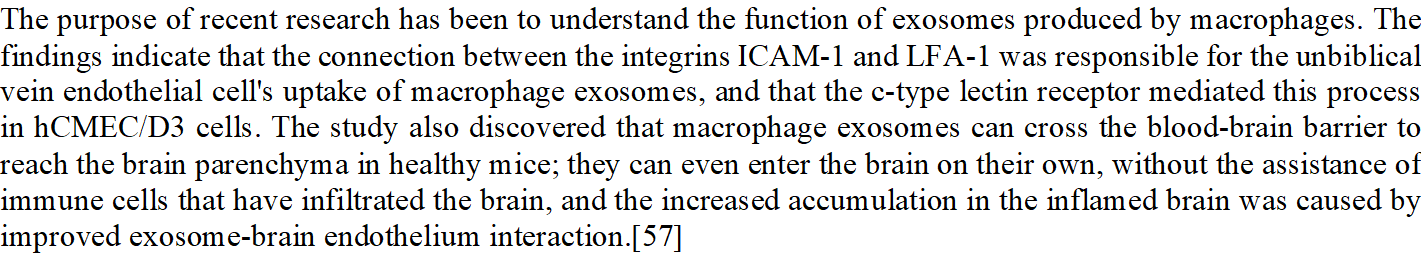


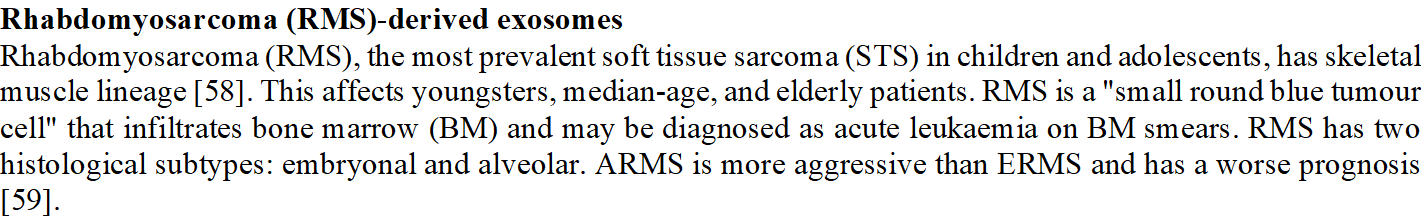
**3.7 Derived Exosomes**

Progenitor cells determine exosome functions. Exosome roles have been studied extensively.

**Macrophage-derived exosomes**

Since their discovery, macrophages have been recognised for their phagocytic activity in the immune system of the body [55]. As they identify and eradicate harmful microbial products and tumour cells, they play a critical part in the prevention of the progression of many diseases [56]. They contribute significantly to heart damage both as an inflammatory component and as a primary regulator.



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Rhabdomyosarcoma cells release exosomes. Exosomes can transport cancer signalling network-related miRNA. RMS-derived exosomes increase human recipient fibroblast and RMS cell proliferation. Exosomes enhance angiogenesis and normal human fibroblast migration and invasion. [60].

****Exosomes affect tumour growth, angiogenesis, invasion, metastasis, and cell motility. They enhance cell development, adhesion, and polarity [61]. Aggressive cancer cell exosomes can impact endothelial cells and metastatic tumour morphology and function. Thrombin-exporting exosomes mediated intracellular communication. Exosomes trigger recipient cell RhoA/Rock pathway [62]. Exosomes modulate host stromal responses to create a protumorigenic or antitumorigenic environment [63].

****Malignant mesothelioma, which develops from uncontrolled cellular growth of mesoderm tissue lining the chest, heart, lungs, abdominal cavity, and intra-abdominal organs, is rare [64]. Asbestos-related mesothelial pleural or peritoneal cancer is aggressive and has a poor prognosis. Malignant mesothelioma is fatal and difficult to treat due to its low occurrence, resistance to most chemotherapies, and tumour intricacy. The 5-year survival rate for peritoneal mesothelioma is less than 15%, and pleural tumour survival is less than 1 year [65].

Human malignant mesothelioma cell exosomes immune-regulate cancer progression. They govern tumour microenvironment recipient cells and include metastatic factors. MM-derived exosomes contain 111 immunoregulation-related proteins, 26 of which were found in mEXOS, including OSMR, ABCC1, and SAE1. OSMR, a multifunctional cell surface cytokine receptor, promotes malignancy by increasing cell motility, invasiveness, and angiogenesis [66].

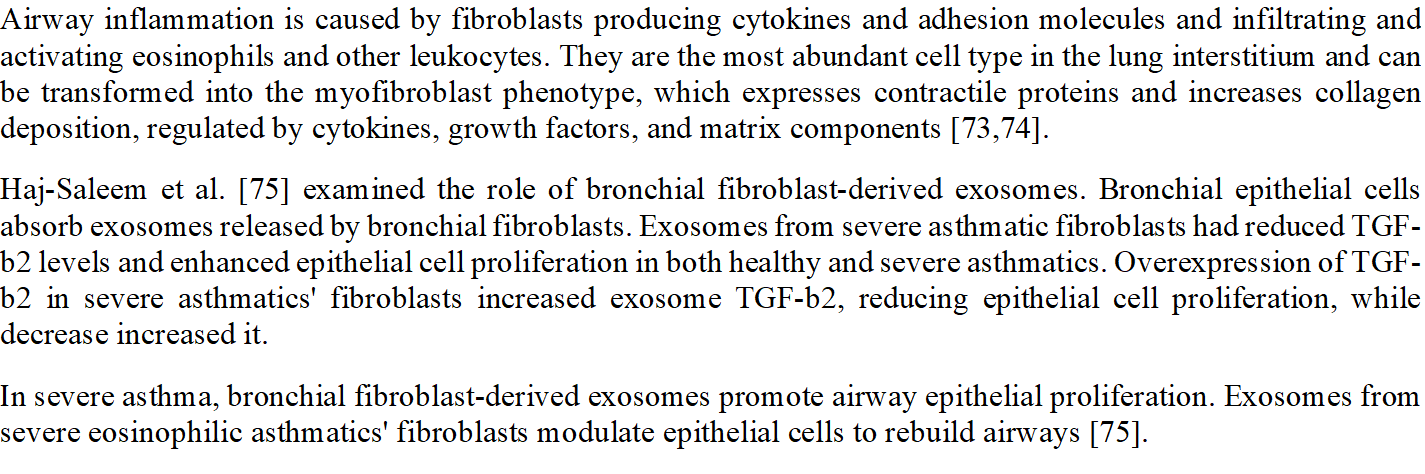
**Osteoclast-derived exosomes**

Hematopoietic/monocytic lineage osteoclasts are large multinucleated cells that specialise in bone resorption and bone remodelling [67]. They are tightly coupled to primary osteogenesis cells (osteoblasts). MCSF and RANKL from osteoblasts and osteocytes influence myeloid precursors to become osteoclasts. Osteoprotegerin (OPG) from the osteoblast lineage inhibits osteoclast development. Osteoclasts polarise proteolytic enzyme and acid secretion, degrading bone. They hydrolyze and solubilize bone organic and inorganic components [68].

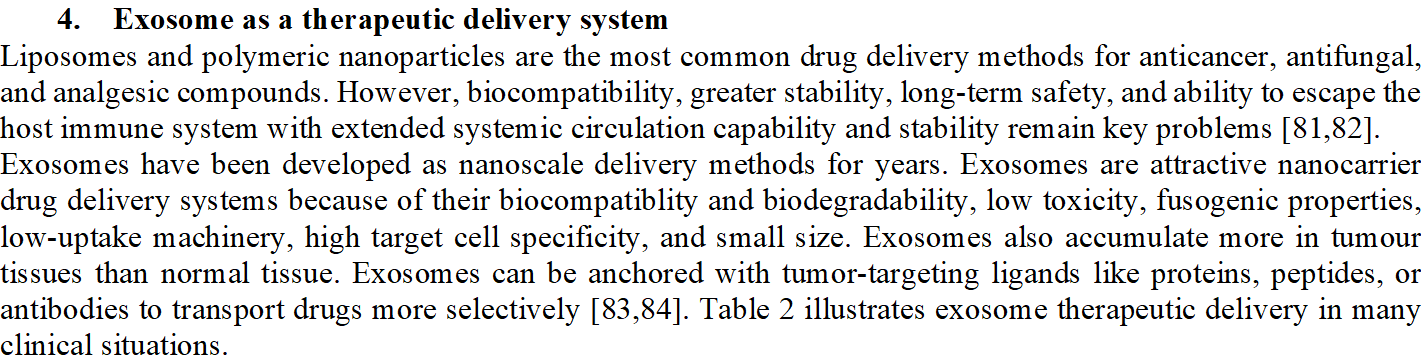
Exosomes release miR-214 to decrease osteoblast activity during osteoclastogenesis. Osteoclast effects on osteoblasts are reduced by exosome inhibition. Cell recognition molecules on exosomes help recipient cells target and absorb them. MiR-214-containing exosomes inhibit osteoblasts via linking. Osteoclast-derived ephrinA2 acts on osteoblasts through its Eph receptor [69].

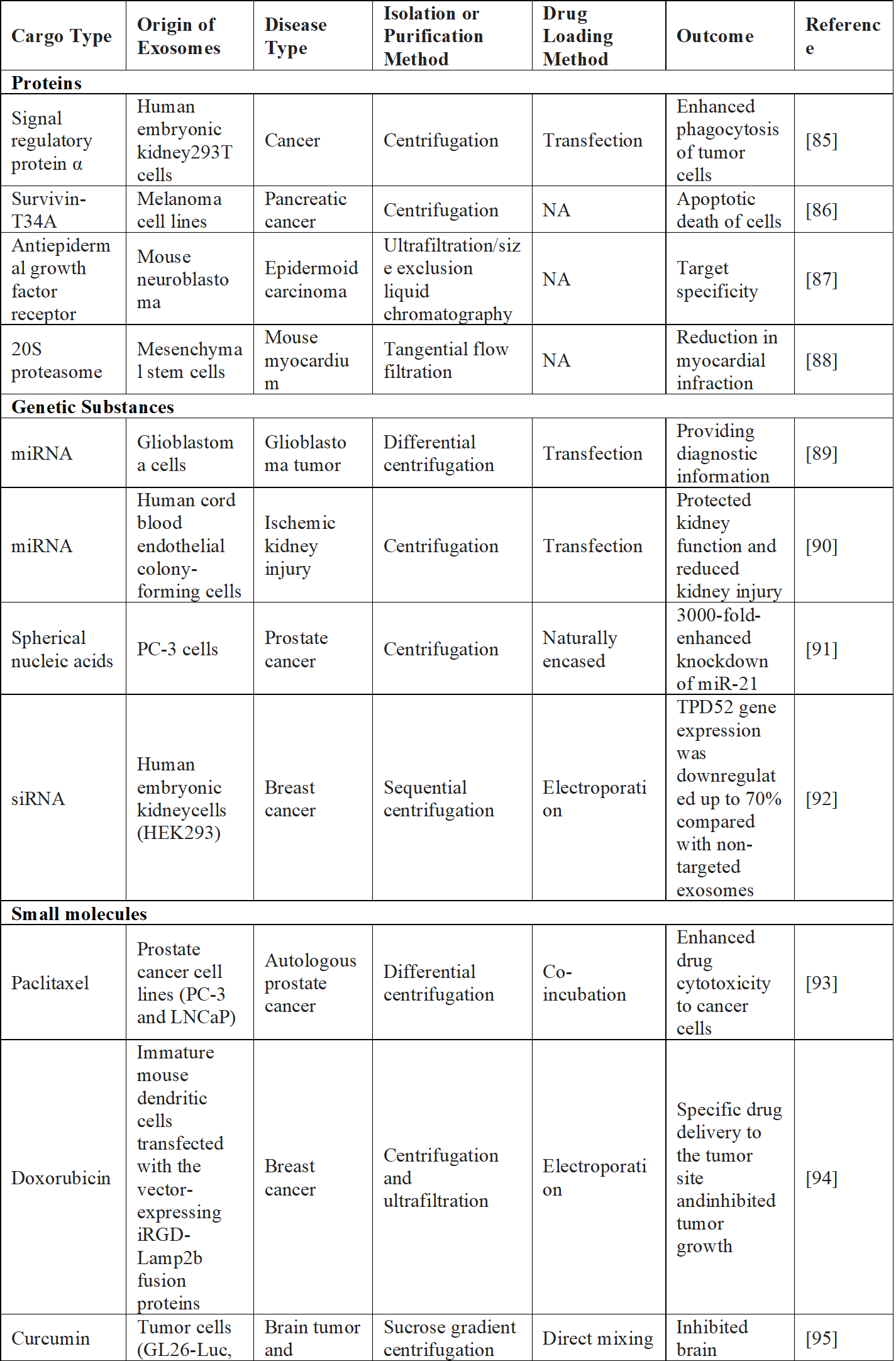
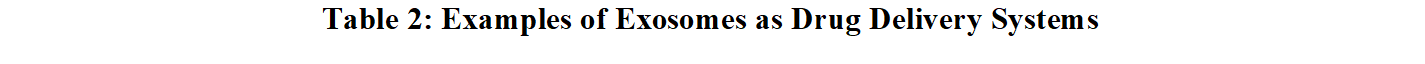
****Smoking, obesity, frequent drinking, and a family history of pancreatic cancer might cause it [70]. Pancreatic cancer is difficult to treat due to its rapid progression, lack of early symptoms, and low response to standard therapy. Pancreatic cancer cells (PCCs) release exosomes to communicate [71].

Cancer cells alter nearby and distant cells via secreting exosomes. PCC-derived exosomes enhance cancer cell proliferation and migration. PCC-derived exosomes also activate and profibrogenize pancreatic stellate cells [72].

********MSC are heterogeneous stromal regeneration cells that can be collected from different adult organs [76,77].

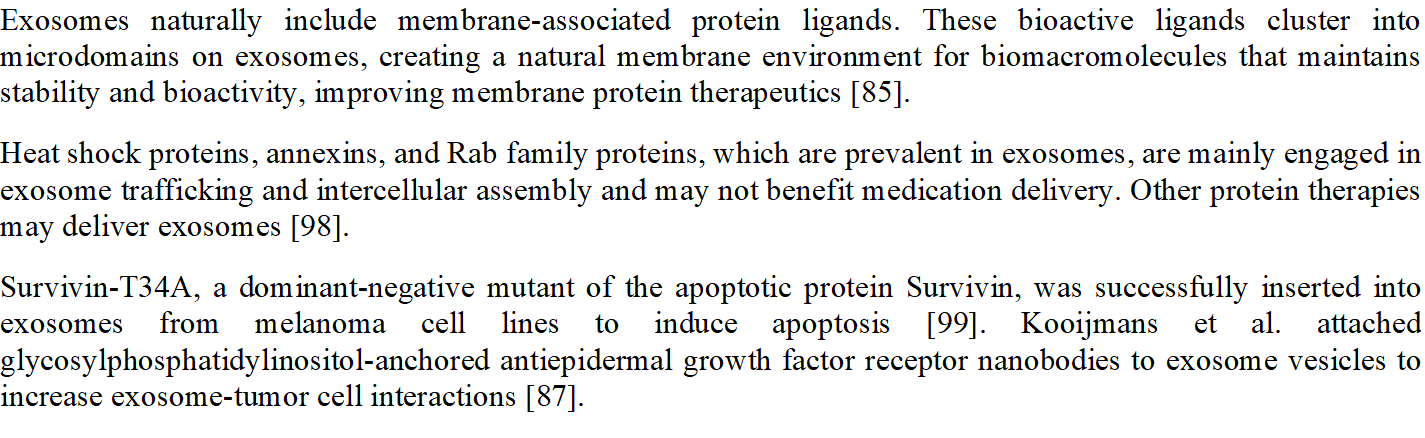
MSC-derived exosomes transport nucleic acids, proteins, and lipids. MSC-derived exosomes, rich in proteins and RNAs, can maintain tissue homeostasis and respond to external stimuli. Another study found MSC-derived exosomes may treat liver disease. MSC-derived exosomes may protect against myocardial I/R injury by anti-apoptosis, cardiac regeneration, cardiac remodelling, anti-inflammatory effects, neovascularization, and anti-vascular remodelling [78].

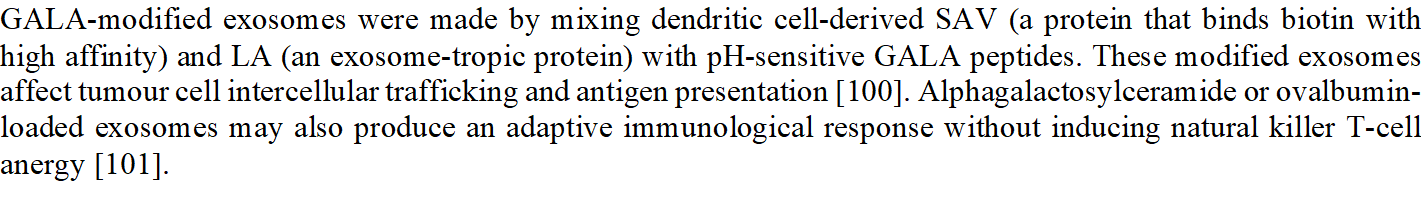
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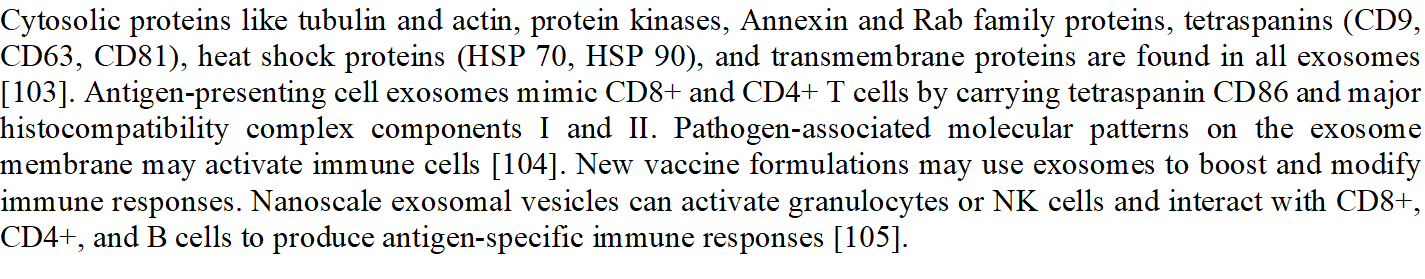
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**4.1. Protein and Peptide Delivery**

Protein or peptide delivery using exosomes is advantageous. Exosomes were initially studied as a garbage bin collecting cell-unwanted proteins, lipids, and nucleic acids. Exosomes have carried biological substances for diagnostic and therapeutic reasons for years. Most cells' exosomes carry endogenous protein molecules, suggesting they could transfer proteins or peptides. Exosomes deliver enzymes, transmembrane, and cytoskeletal proteins. Exosome vesicles can carry macromolecules like lipids, proteins, and genetic material from mother cells to neighbouring cells [97].



Tian et al. [102] attached exosomes to the c(RGDyK) peptide utilising bio-orthogonal chemistry to cure ischemic stroke by targeting the brain lesion. These modified exosomes also contained curcumin to reduce inflammation and cellular death in the lesion. cRGD–exosome administration showed promising therapeutic effectiveness and targeting in vivo.

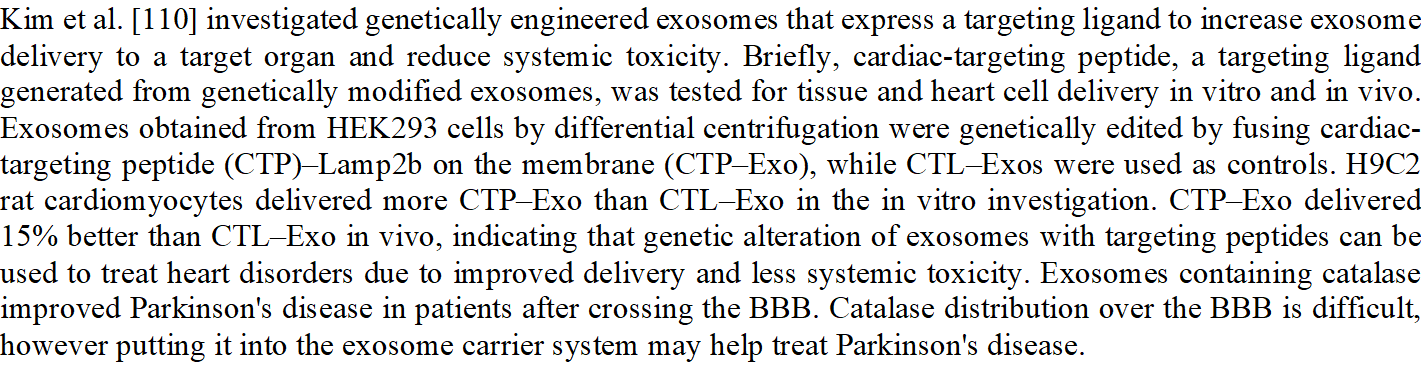
Sandra et al. [106] examined exosomes as vaccine adjuvants. LPS-stimulated THP-1 human monocytic cells produced exosomes. HBsAg-loaded poly--caprolactone–chitosan nanoparticles and isolated exosomes were homogenised. Exosomes containing HBsAg generated a humoral immune response similar to the control group. Their study suggests that exosomes co-ingested with antigens may increase vaccination immune responses.

Exosomes were tested for neuronal recovery following ischemic stroke by Liu et al. [107]. To target the BBB, enkephalin-tar-exo exosomes were created. In a transient middle cerebral artery occlusion–reperfusion scenario in rats, enkephalin-tar-exo penetrated the BBB and lowered lactate dehydrogenase, p53, and caspase-3. The enkephalin-tar-exo system also increased brain neuron density and neurological score following stroke.

Trastuzumab–emtansine was delivered to HER2-positive malignancy by Barok et al. [108]. Ultracentrifugation and trastuzumab–emtansine therapy identified exosomes from HER2+ (SKBR-3 and EFM-192A breast cancer), HER2 (MCF-7 breast cancer), and gastric cancer (SNU-216) cell lines. Trastuzumab–emtansine bound to HER2+ cancer cells via antibody–drug-conjugated exosomes, inhibiting proliferation and activating caspase-3.

Cho et al. [85] compared the efficacy of exosomes and the ferritin nanocage carrier in signal regulatory protein α delivery. Because macrophages phagocytose tumour cells more than nanocages, exosomes inhibited tumour growth more than nanocages. Exosome vesicles may have an advantage over alternative delivery platforms because their abundance of proteins and lipids creates a perfect membrane protein activity and distribution milieu.

Exosomes' assembly, binding, fusion with targeted cells, and interactions with the extracellular matrix are complex. For example, paraformaldehyde-mediated crosslinking of proteins on exosome surfaces decreased fusion with parental cells by 20%. Exosomes solubilized with octylglucoside and rebuilt by dialysis to remove membrane proteins were less able to merge with target cells [109]. Exosomes with decreased proteins showed comparable fusion efficacy to large unilamellar vesicles with lipid compositions identical to natural exosomes, indicating the role of exosome-associated proteins in fusion events.

Haney et al. [111] used incubation with or without saponin permeabilization, freeze–thaw cycles, extrusion, and sonication to integrate catalase into exosomes. Western blot examination showed that extrusion and sonication best incorporate catalase into exosomes. To validate BBB catalase delivery, exosomes were labelled with lipophilic fluorescent dye. Pheochromocytoma (PC12) cells were treated with fluorescent exosomes. Confocal pictures showed PC12 cells absorbing labelled exosomes. In in vitro-activated macrophages, exosomal catalase neutralised reactive oxygen species (ROS).

**4.2. Exosomes in Gene Delivery**

 Exosomes deliver DNA and RNA to targeted cells, causing genetic changes in physiologic and pathological processes. Exosomes are used as medication delivery vehicles to deliver therapeutic genetic elements that change gene expression in certain disorders and improve genetic therapy.

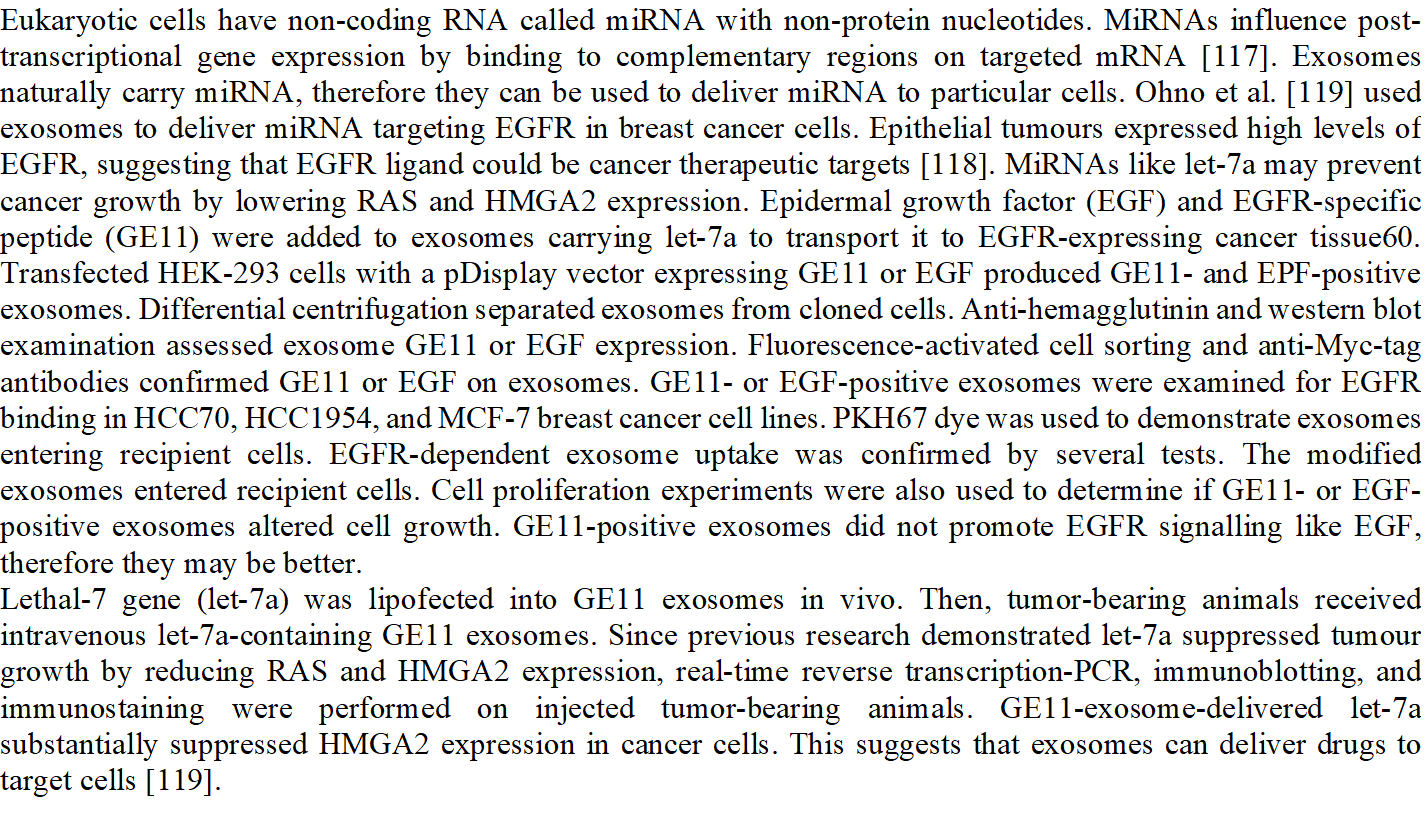
**4.2.1 Small interference RNA (siRNA)**

Genetic treatment disrupts genes with siRNA. However, these siRNA breakdown swiftly in the systemic circulation. However, exosomes protect and distribute siRNA to targeted cells. Exosomes may deliver exogenous siRNA, but additional research is needed. The first published study, by Alvarez-Erviti et al.[112], used exosomes to deliver siRNA to mouse brains. Another work used human exosomes to deliver siRNA to T and monocytes [113]. Safe, effective, and target-specific genetic material delivery vehicles are scarce. That study sought to deliver gene therapy vectors. Exosomes are non-immunogenic and naturally transfer RNA between cells. The study sought a gene therapy vector delivery mechanism.

Differential centrifugation was used to isolate exosomes from healthy donor peripheral blood, TB-177 lung cancer cells, and HeLa cells. Chemical transfection and electroporation introduced siRNA into exosomes. Chemical transfection was ineffective and inconclusive. Western and northern blotting, confocal imaging, and flow cytometry revealed siRNA was introduced into exosomes. Plasma exosomes were electrophoretically transfected with Alexa Fluor 488-tagged siRNA against mitogen-activated protein kinase 1 (MAPK-1) and co-cultured with healthy donor peripheral blood monocytes and lymphocytes. Exosomes fluoresced siRNA in recipient cells' cytoplasm. Flow cytometry confirmed siRNA distribution to PBMC. Immunoblotting assessed whether exosome-delivered siRNA produced post-transcriptional gene-silencing in recipient cells. Exosomal siRNA downregulated MAPK-1 expression, indicating gene silence. The study proved that exosomes can deliver genetic therapy [57].

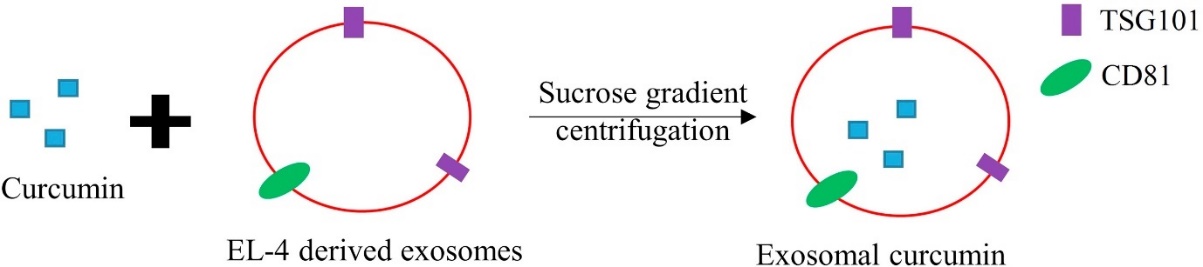
Exosomes delivered siRNA to human cells in another in vitro investigation. RAD51, a eukaryotic gene protein that repairs DNA double strand breaks, may suppress cancer cell proliferation [114]. Exosomes-delivered RAD51 siRNA worked in human cells in an in vitro investigation. After ultracentrifugation, HeLa exosomes were chemically loaded with Alexa-flour 488-labeled siRNA and co-cultured with recipient cells (HeLa and HT1080 cells). Confocal microscopy and flow cytometry demonstrated exosome siRNA delivery. The study also examined siRNA efficiency and RAD51/RAD52 downregulation. Western blot examination indicated a significant decrease in RAD51 and RAD52 protein levels, confirming gene downregulation [115]. These findings demonstrated exosomes' therapeutic potential by maintaining cargo function. Exosomes from different cell types have slightly varying compositions and functions. Endothelial exosomes cause vascular inflammation and atherosclerosis. Exogenous content delivery is unknown. Another work used endothelial exosomes to deliver siRNA to endothelial cells [116]. Exosomes were extracted from endothelial cells by filtration and ultracentrifugation and tested for interactions. Electroporation was used to load endothelium exosomes with siRNA. Luciferase-expressing endothelial cells were treated with siRNA-loaded exosomes. siRNA silenced a vector expressing luciferase (pGL2) in transiently transfected endothelial cells. Endothelial exosomes with siRNA had considerably lower luciferase expression than controls. In vitro, endothelial exosomes can carry foreign substances to cells and operate at the intended spot.

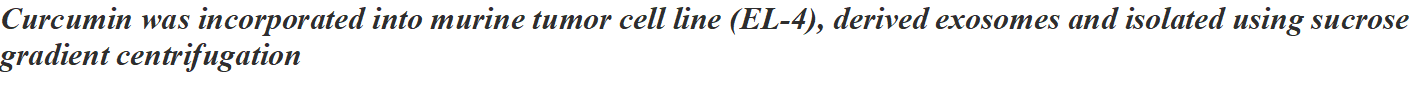
**4.2.2. Non-Coding RNA (miRNA)**

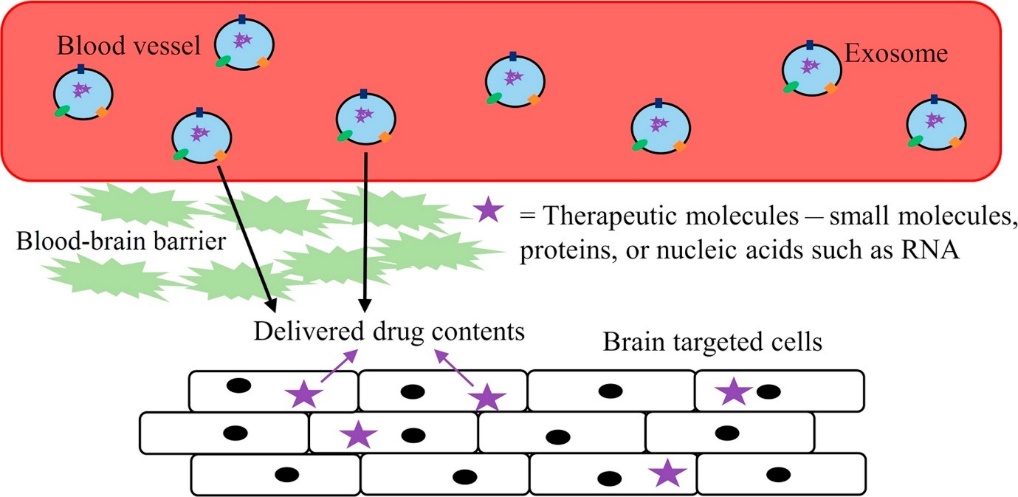
**4.2.3 Small molecules**

Therapeutic medication delivery using exosomes has been extensively studied. Exosomes delivered curcumin to cure an inflammatory disease53. Turmeric rhizomes contain curcumin, a polyphenol with anti-inflammatory, antineoplastic, antioxidant, and chemopreventive effects [120]. Curcumin is enhanced by complexing with exosomes. Cancer patients have benefited from clinical trials. Hydrophobicity and preferential interaction with lipid membranes decrease its solubility and bioavailability [121].

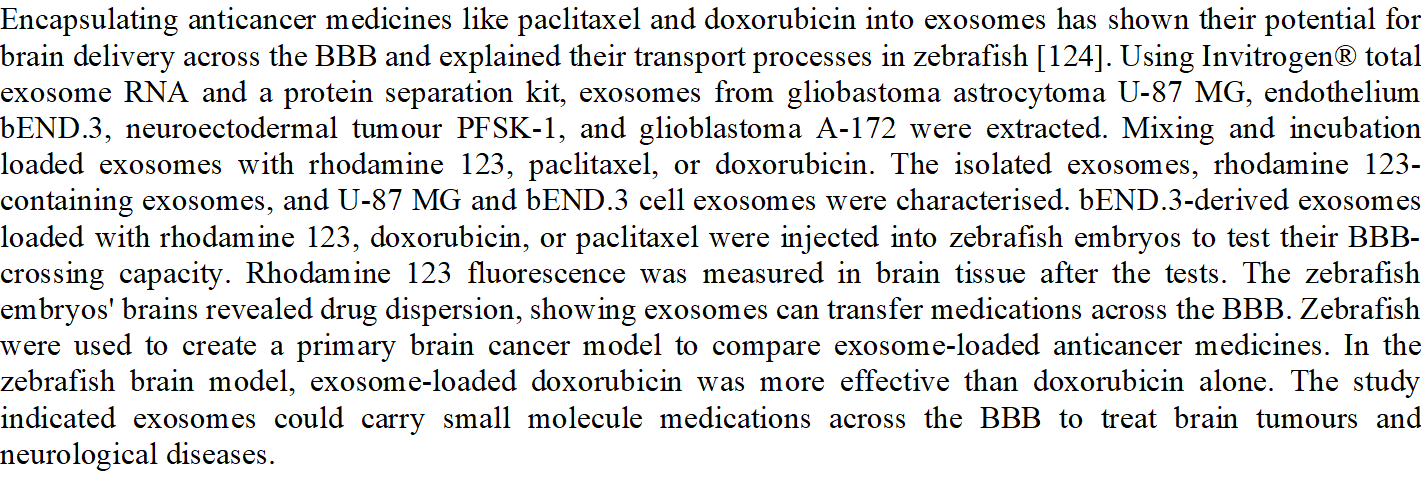
This work used sucrose-gradient centrifugation to integrate curcumin into exosomes from EL-4 mouse tumour cells. TSG101 and CD81 were employed to identify the exosome curcumin complex (Fig. 6). Curcumin was incorporated into the exosome and increased in solubility, stability, and bioavailability in subsequent trials. Exosomal curcumin was tested for anti-inflammatory effects in vitro and in vivo. In vitro, macrophages treated with exosomal curcumin generated less inflammatory cytokines like IL-6 and TNF-α than those treated with curcumin alone, showing that it increases anti-inflammatory activity. In an LPS-induced septic shock animal model, exosomal curcumin improved survival relative to curcumin alone. Finally, exosomal curcumin reduced CD11b+Gr-1+ cells, which promote lung inflammation and LPS-induced septic shock53. Exosomes can contain hydrophobic medicines like curcumin, boosting their anti-inflammatory properties.

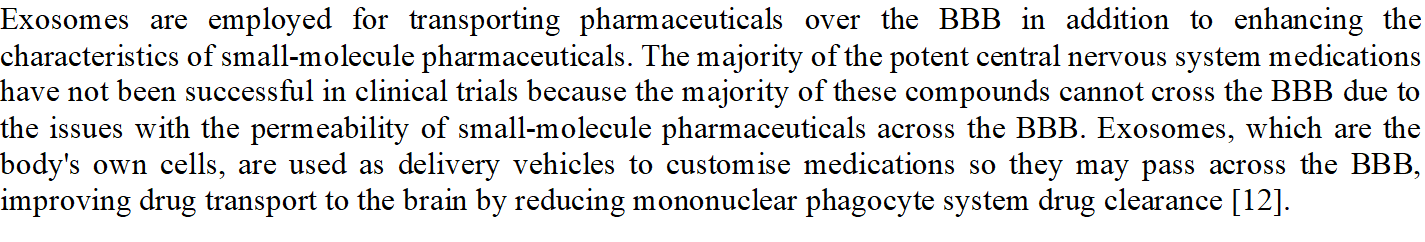


******Exosomes transport tiny molecular drugs across the blood–brain barrier (BBB) and enhance therapeutic characteristics. 98% of potent central nervous system medications cannot penetrate the BBB, and their conceptual potency in labs has failed in clinical trials [122]. Drug permeability through the BBB has been addressed by many nano-formulations. Nano-toxicity and MPS medication clearance are also issues [123]. PEG reduces MPS medication absorption to address these issues. This decreased target cell contact and cerebral drug distribution68, 69. Exosomes, a natural component of the body, can be modified to cross the BBB and improve medication delivery to the brain by lowering MPS drug clearance (Fig. 7).

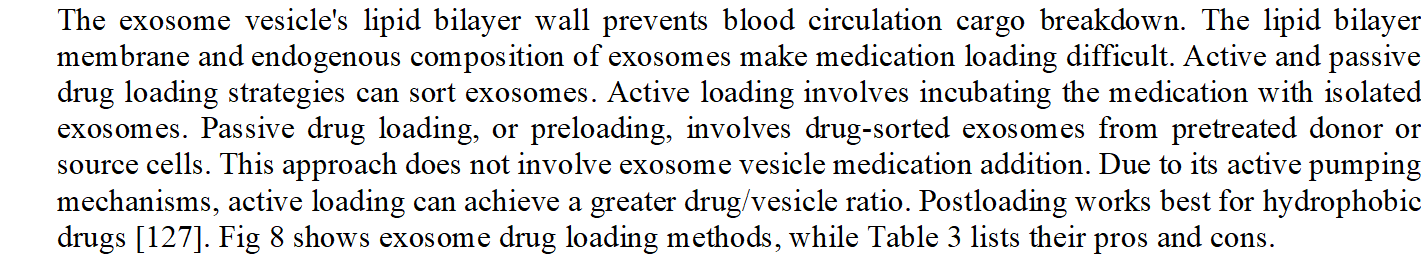


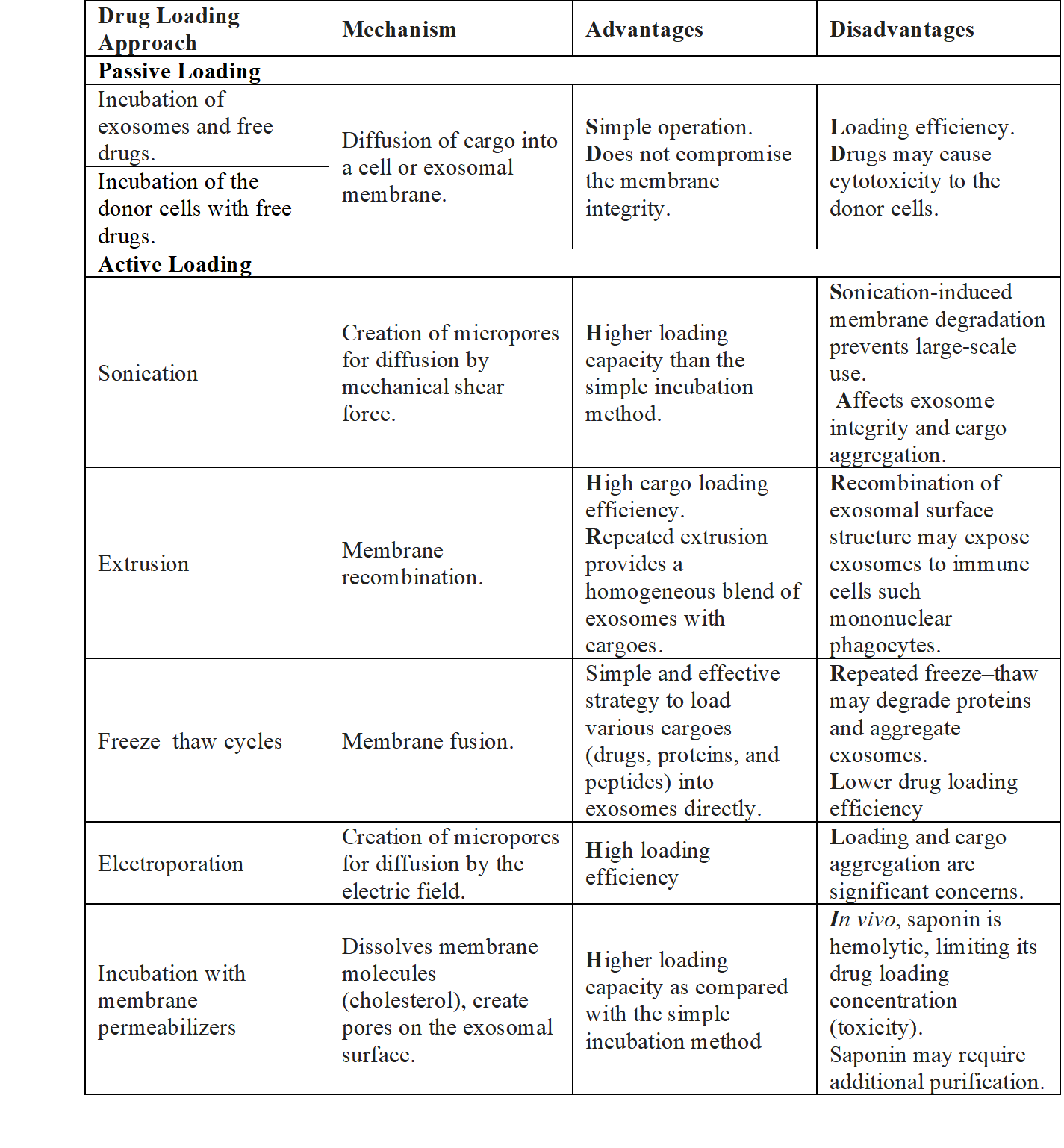
**Fig 7. Ability of exosome and its drug contents to cross the blood–brain barrier.**

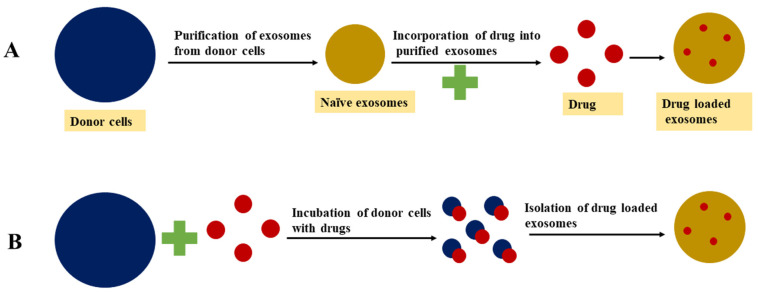
The researchers avoided phagocytosis, cell membrane fusion, and lysosome engulfment by using small, native exosomes. The immunological reaction was modest since exosomes are bodily products. Due to nonspecific tissue targeting and a short half-life, most chemotherapeutic medicines, including doxorubicin, have limited solubility and toxicity and poor efficacy. Exosomes delivered doxorubicin to a mouse tumour tissue model in vitro and in vivo [125]. Exosomes were extracted and purified from pEGFP-C1-RVG-Lamp2b plasmid-containing immature murine dendritic cells by centrifugation and ultrafiltration. Electroporation loaded exosomes with doxorubicin for nanoparticle tracking analysis. Encapsulated exosomes were fused with human breast cancer MDA-MB-231 cells to assess doxorubicin delivery. Fluorescence and overlap demonstrated exosome transport inside cells. These exosomes inhibited in vitro cancer proliferation in MDA-MB-231 by measuring cell viability with cell counting kit-8 (CCK-8). Doxorubicin-encapsulated exosomes inhibited cells similarly to free doxorubicin, demonstrating their anticancer properties. Injecting fluorescent exosomes tested their ability to deliver doxorubicin to tumour tissue and prevent tumour growth in vivo. The data demonstrated doxorubicin-containing exosome accumulation at the targeted organ and significant tumour growth suppression. The results showed exosomes efficiently target and deliver doxorubicin.



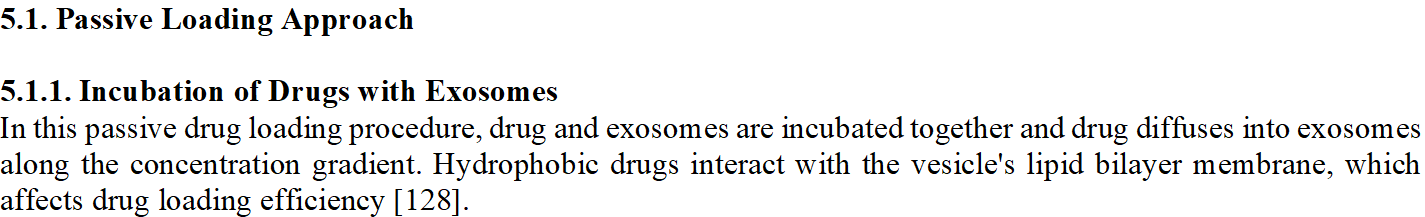
1. **Exosomes Drug Loading Techniques**



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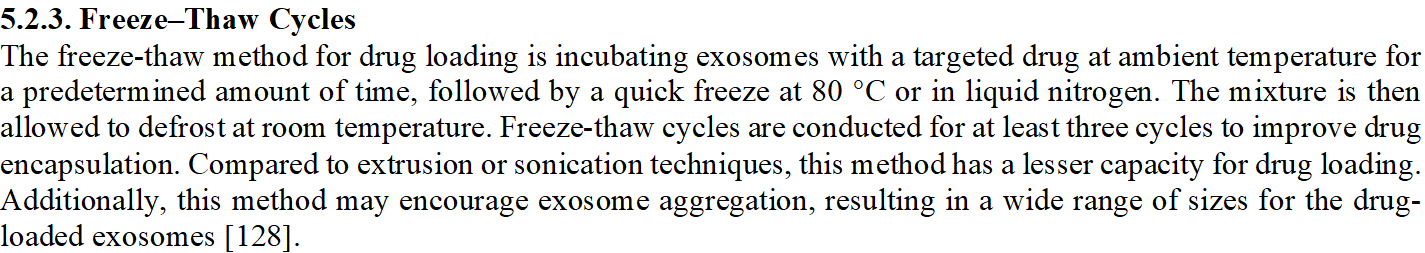
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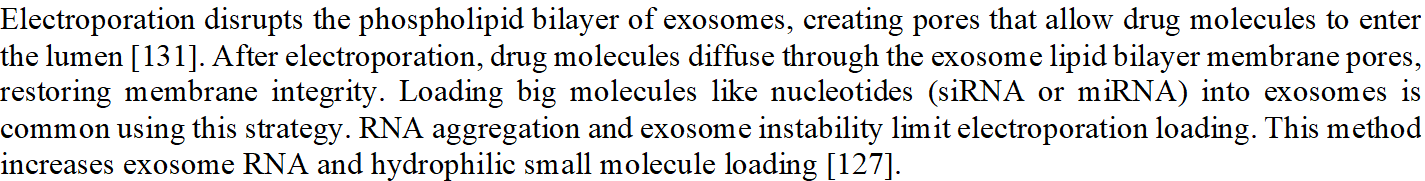
****This method treats selected exosome donor cells with a drug molecule and secretes drug-loaded exosomes. Donor cells acquire bioactive or therapeutic chemicals and release exosomes that may retain them. This untargeted method may yield few exosomes [128].

****Active medication loading temporarily disrupts the exosome membrane so active cargo can diffuse into the vesicles. Loading the required chemicals into exosomes restores membrane integrity. Sonication, extrusion, and freeze–thaw cycles break exosome membranes [129]. Active drug loading increased exosome vesicle drug loading capacity by 11 times compared to passive loading [127]. This method risks damaging exosome targeting characteristics and native structure during membrane disruption [129].

****Homogenizer probes sonicate donor or target cell exosomes with a drug or protein of interest. Sonication deforms the exosome membrane and permits bioactive chemicals to permeate in [128].

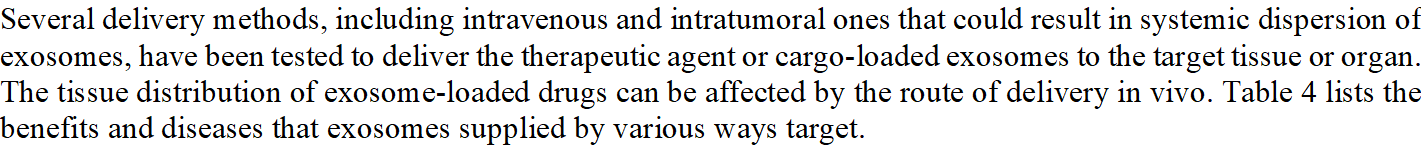
**** Extrusion is a drug loading technique that uses a syringe-based lipid extruder. Exosomes that have been extracted from donor cells are combined with a specific medication and fed into a syringe-based lipid extruder with a membrane that has pores between 100 and 400 nm at a specific temperature. The medication and the damaged exosome membrane are forcefully mixed together during extrusion [130].

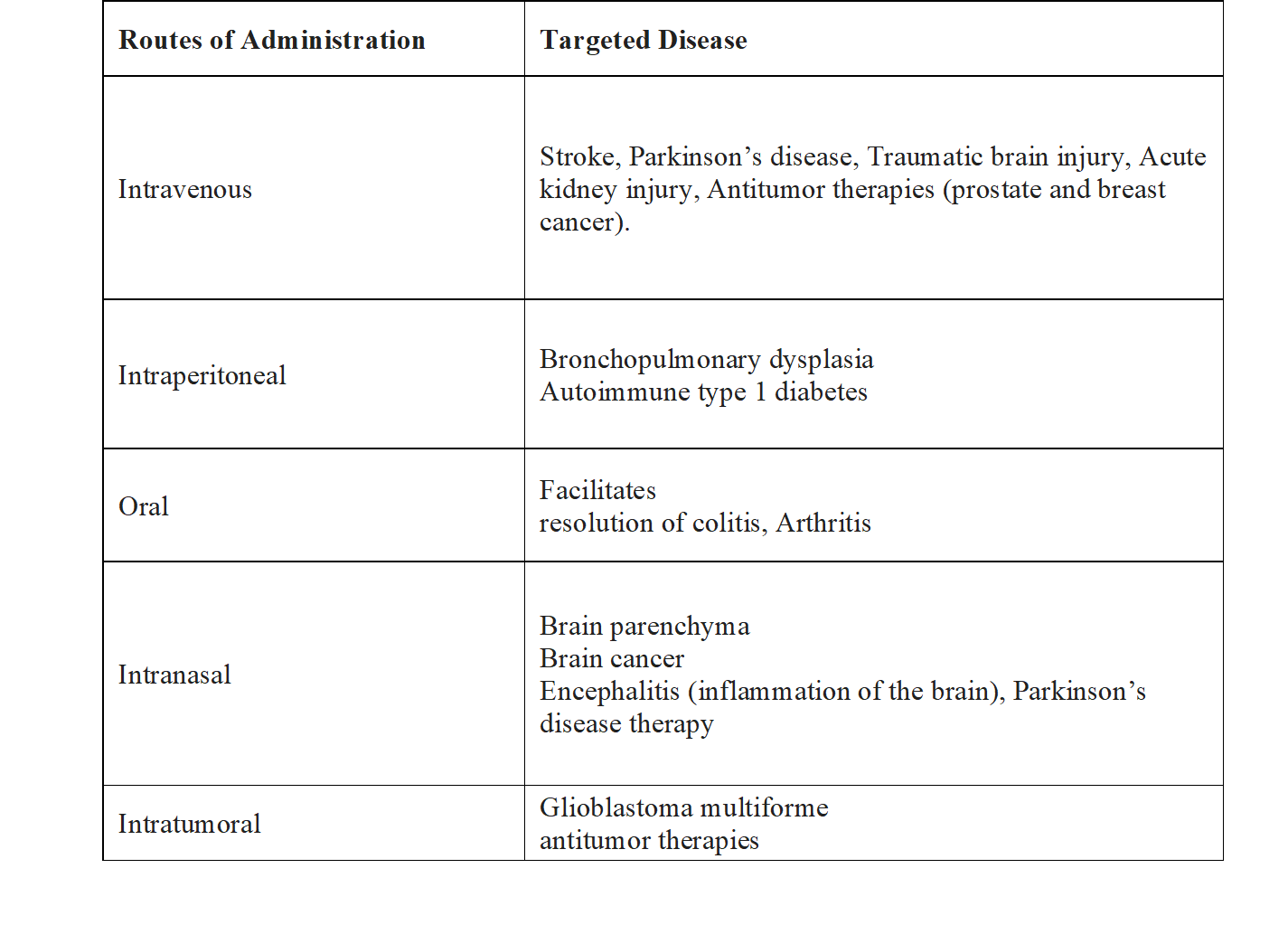
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****Exosomal membrane permeability is caused by interactions between surfactants and membrane permeabilizers with the cholesterol in cell membranes, which result in the formation of pores. The membrane permeability method can improve the catalase loading efficiency into exosomes in comparison to the incubation method [132]. An earlier study found that the passive loading method without saponin resulted in an 11-fold increase in drug loading of hydrophilic compounds into exosomes [127]. This technique calls for exosomes to be isolated following an adequate amount of saponin incubation for drug loading.

1. **Exosomes Administration Routes**



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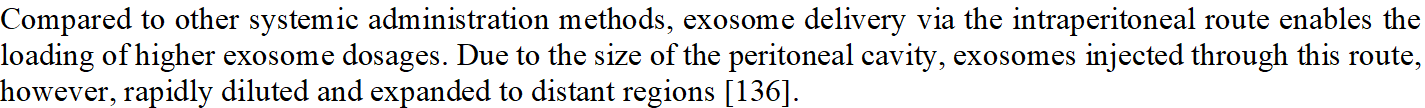
**6.1. Intravenous Administration**

Exosome-based medication administration should bypass immune cell and hepatic clearance because it is endogenous. Intravenous exosome-loaded medicines can reach the brain, pancreas, and tumour tissues [133]. Intravenous injection of exosomes may favour extravasation and retention inside solid tumours due to poor lymphatic drainage and leaky blood arteries [131]. Thus, exosomes should be given intravenously, especially in cancer. After intravenous injection, exosomes had a half-life of 2 minutes in systemic circulation and were barely detectable after 4 h [134]. The liver and lungs accumulate exosomes, suggesting that they are cleared from systemic circulation like liposomes. Intravenous delivery permits exosomes to reach the target region, although their short half-life in circulation is a drawback [134]. However, PEGylation of exosome particles can prolong their circulatory half-life and prevent fast clearance following intravenous injection.

**6.2. Intratumoral Injection**

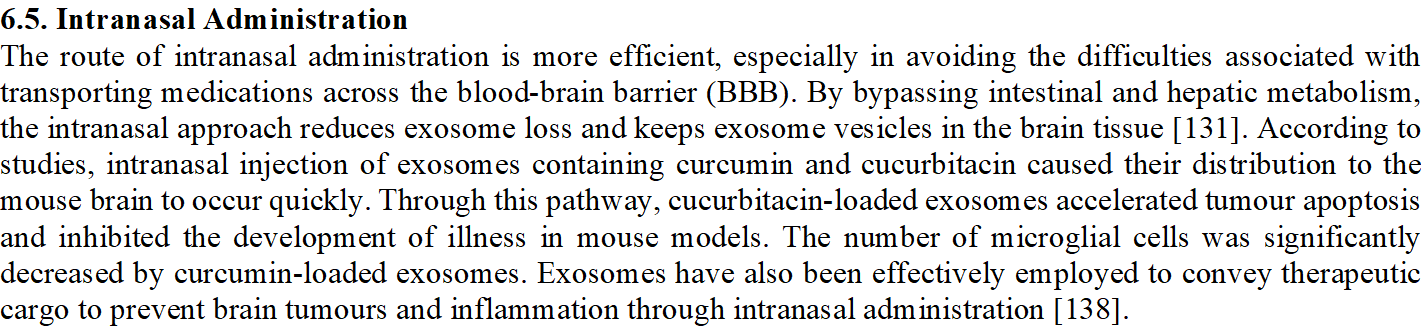
Exosomes laden with a therapeutic drug can be injected intratumorally for some cancer types so that the tumour can be reached without the need for significant invasive manipulation. Following intratumoral injection of exosome-loaded therapeutic cargo to the tumour mass, prior investigations have documented decreases in tumour volume or dimensions [135]. The benefit of this strategy is that the treatments can be delivered specifically to tumour cells by directly injecting exosomes [131].

**6.3. Intraperitoneal Route**



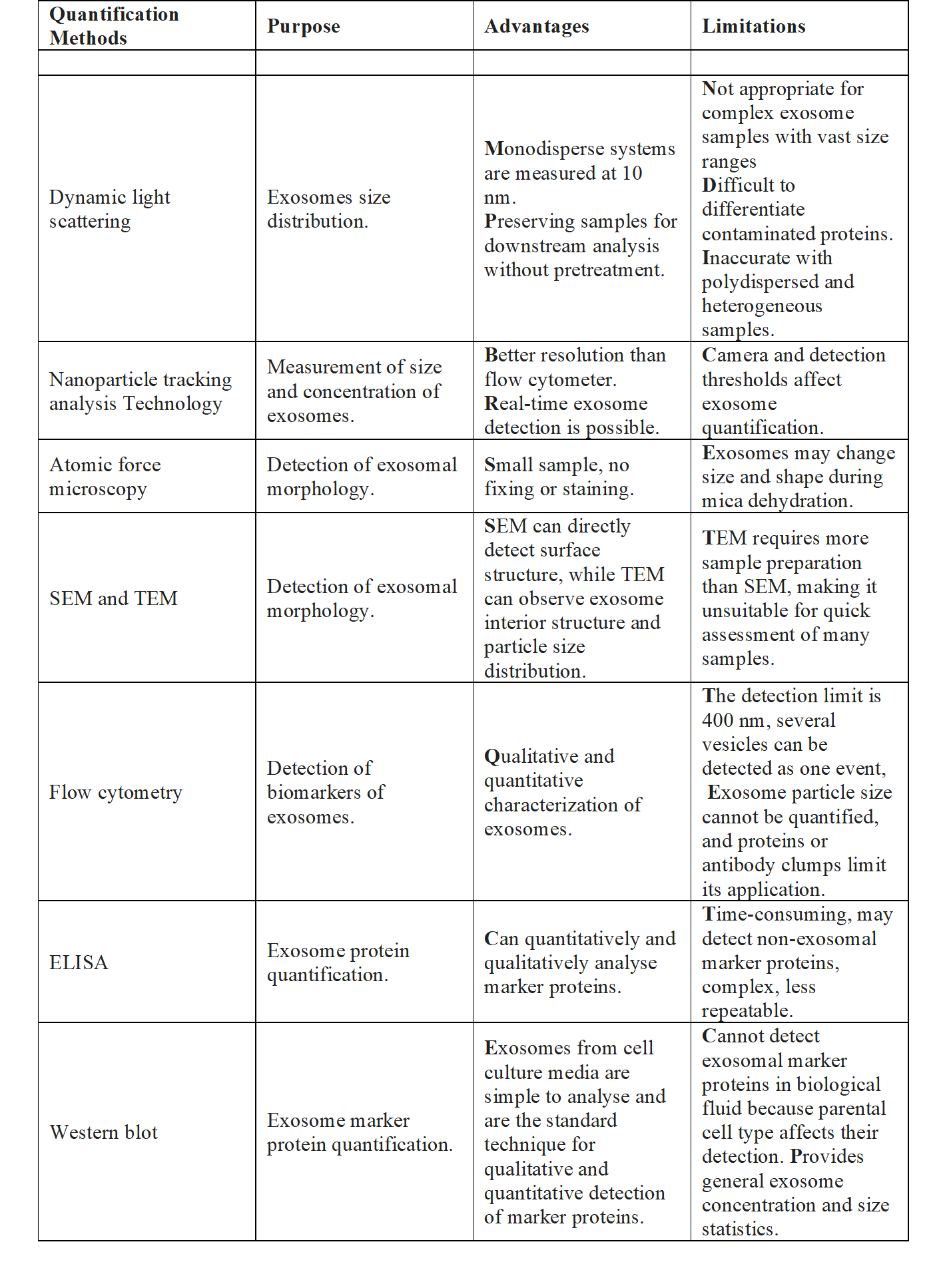
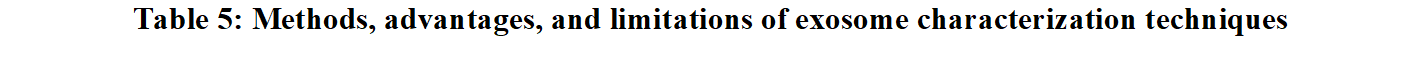
**6.4. Oral Administration**

Exosome delivery via oral administration entails a number of challenges, including enzymatic activity, changes in the pH, and changes in the intestinal barrier along the gastrointestinal system, despite the fact that it is convenient, simple, and helps patients comply with treatment. Intestinal microbiota traits and the presence of significant acid-base fluctuations are obstacles that must be overcome for exosomes to reach the target tissue of interest. Exosome distribution via oral ingestion is more effective at reaching intestinal luminal epithelial surfaces than non-gastrointestinal tissues [136]. Paclitaxel was delivered orally using exosomes produced from bovine milk by Agrawal et al. [137] for increased efficacy and less toxicity. Exosomes containing paclitaxel displayed remarkable stability when simulated gastrointestinal fluids were present. Significant tumour growth suppression was seen against human lung tumour xenografts after oral administration in nude mice. Oral administration of paclitaxel-loaded exosomes reduced systemic toxicity and inflammation in comparison to intravenous therapy.

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1. **Characterization Techniques**

Exosome samples should be properly characterised after they have been isolated in order to validate the isolation technique. The accuracy of the methods used to measure the quantity and purity of exosomes is one of the main problems in exosome biology. Marker-based, biophysical, and imaging-based methods make up the three categories of characterization techniques used to gauge exosome purity. Table 5 lists the benefits and drawbacks of exosome characterisation methods.

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**7.1. Imaging**

Qualitative imaging tools determine exosome morphology. Exosome vesicles are too large for microscopic imaging. High-resolution exosome imaging is possible with AFM, SEM, and TEM.

**7.1.1. Atomic Force Microscopy (AFM)**

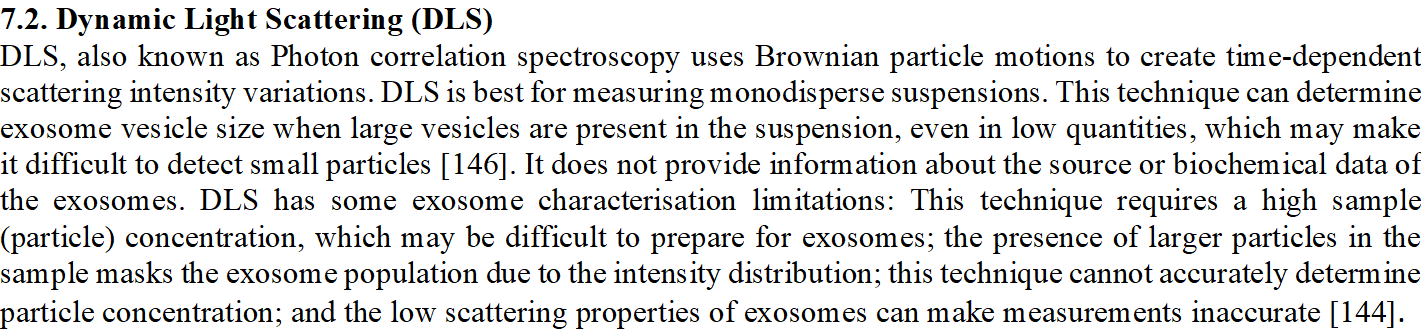
AFM imaging creates a topological map by measuring the force between the probing tip and sample surface. AFM surface scans with a sharp cantilever tip. The cantilever deflects towards the sample surface and delivers sub-nanometer, high-resolution imaging at less than 1 nm [139]. A mica substrate with an exosome vesicle is dried at ambient temperature, then washed and dried in liquid nitrogen. Using a silicon probe and software, the dried material may be seen under AFM [140]. AFM can non-destructively quantify the exosome vesicle in native settings with little sample preparation. This method gives exosome shape, biomechanics, and biomolecular data. Several investigations have used AFM to characterise cell-derived exosome membrane composition, mechanical characteristics, morphologies, and sizes [141].

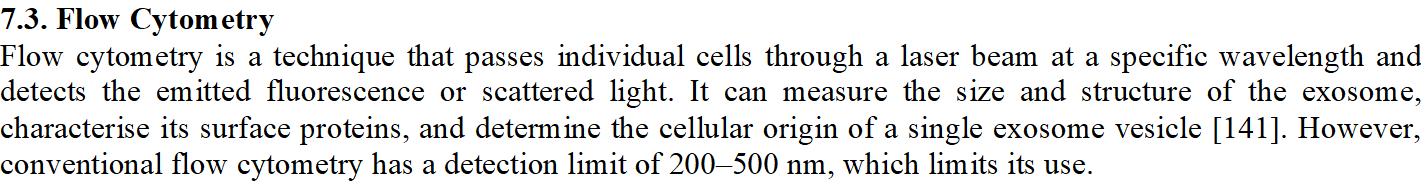
**7.1.2. Transmission Electron Microscopy (TEM)**

TEM is used to detect exosomes in solution and assess their quality by studying their structure, size, and morphology [142]. TEM determines structure and morphology using an accelerated electronic beam with a shorter wavelength than light. The image is formed by a stream of electrons passing through a material, generating a secondary electron [141]. Exosome vesicles are treated with paraformaldehyde (2% w/v) and placed on formvar–carbon-coated grids for 20 min. After washing with PBS, the carbon-coated grids are incubated with glutaraldehyde, a crosslinking agent, and rinsed. Finally, exosome vesicles are dyed with uranyl acetate solution (2% w/v) and air-dried [143]. Multiple stages and electron beams can modify exosome shape during TEM sample preparation. Thus, Cryo-TEM can be used to reduce sample preparation effects [121].

**7.1.3. Scanning Electron Microscopy (SEM)**

Accelerated electrons in the SEM technique contain a lot of kinetic energy, which is dissipated as various signals via electron sample interactions as the incident electrons decelerate in the solid sample. Exosome samples are glutaraldehyde-fixed and ethanol-dehydrated on a carbon-coated or copper grid. SEM analysis follows air-drying and sputter-coating the grids with gold at 2–10 nm. SEM pictures of exosomes were spherical and bulging [144,145].

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****NTA is based on DLS. In NTA, a microscope captures Brownian motion particles. NTA measures exosome concentration and size distribution from 10 nm to 2μm. Image analysis can follow exosomal particles and estimate hydrodynamic sizes. This method can detect sample particles of varying sizes by imaging a particle in distinct areas. NTA can also identify exosome antigens using fluorescently labelled antibodies. NTA pre- and postprocessing variables, such as camera sensitivity and particle detection threshold, can affect outcomes. NTA performance depends on sample preparation and dilution. NTA is interesting due to its speed, capacity to detect exosomes as small as 30 nm, and ease of sample preparation and recovery in their native state [148].

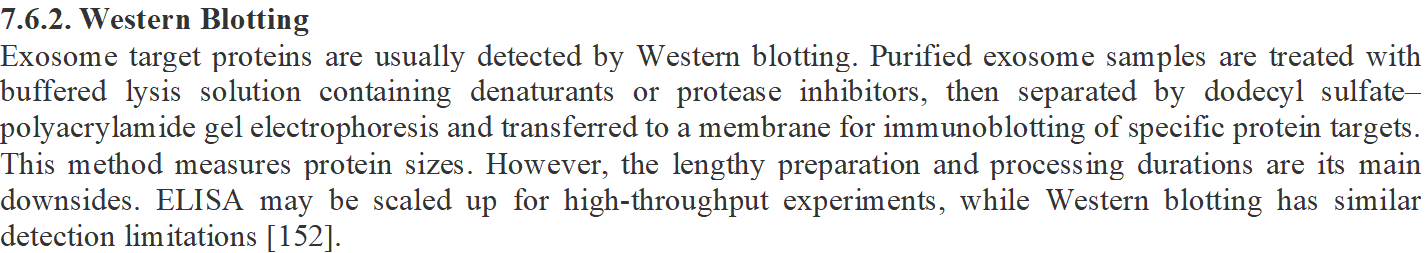
****TRPS biophysically passes single particles through nanoscale pores. Particles passing through holes detect resistance pulse duration and frequency. This determines concentration, size, and zeta potential. TRPS can measure colloidal particles from 50 nm to cell size, which is important for studying cellular functioning and uptake. Exosome size and particle concentration match TRPS better than NTA. Particles obstruct TRPS pores and cause system appropriateness difficulties [149].

**7.6. Protein Characterization**

Protein or marker-based approaches can demonstrate that isolated exosomes have minimal quantities of possible contaminants and exosome signals. Total protein assay measures exosome protein. This approach is difficult to use because exosomes are co-isolated with non-exosomal proteins. Exosomes from most sources contain membrane or intraluminal proteins from endosome development. Western blot or ELISA can characterise protein markers [150].

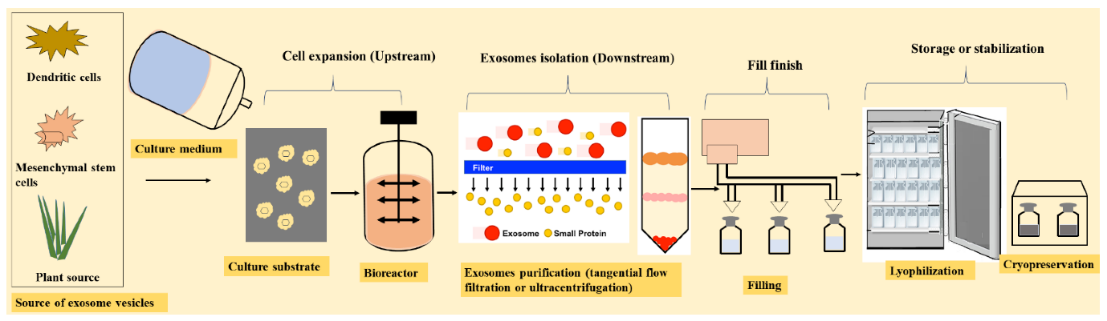
**7.6.1. ELISA**

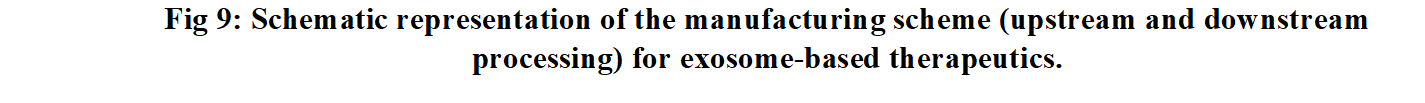
The protein content of the exosomes is detected and measured using the plate-based assay method known as ELISA. Some of this technique's drawbacks include the necessity for a high sample volume and its limited sensitivity. Exosome numbers can be precisely measured using the ELISA method [151].

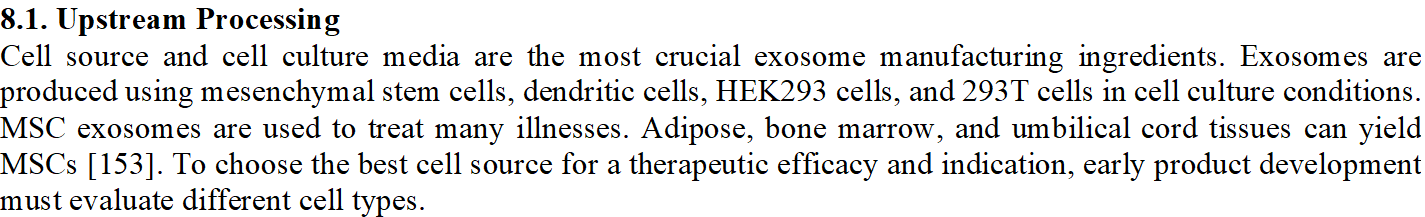
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**8. Manufacturing of Exosomes**

Exosomes, a revolutionary biotherapeutic, are made via cell culture and purification, like biologics. Exosome manufacturing requires culturing the parent cell line, extracting from the conditioned medium, and purifying from process-related impurities as extracellular vesicles. Upstream and downstream pathways make exosomes. Fig. 9 shows the exosome-based drugs production method**.**



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****Cell culture medium isolates and grows the parent cell line. Cell culture media contains FBS and hPL. Dissociation enzymes used in manufacturing classify the cultivation medium as animal-free or animal-derived [154].

Endogenous exosomes in FBS and hPL can contaminate the released exosome product [155]. For high-quality exosomes, use a serum-free medium (SFM). Early medium selection, especially for serum-depleted media, can impact cell line protein concentration and function [156]. Cell culture uses flasks and bioreactors. In a recent investigation, a stand tissue culture flask and CellBIND® surface with a negative surface charge were prepared with an oxygen-containing functional group [157]. Large-scale production using dynamic bioreactors. The bioreactor's cell density, secretion, and reuptake affect exosome yield. T-flask or hollow fibre bioreactors are utilised to make exosomes [158]. Harvesting exosomes requires a hollow fibre bioreactor with a molecular weight cutoff membrane due to their size (60–200 nm). Bioreactor systems provide a dynamic cell culture environment and continuous medium collection for downstream purification [157]. Oxygen, carbon dioxide, temperature, and homogenous feed delivery in the bioreactor can alter exosome quality.

**8.2. Downstream Processing**

Filtration removes cell detritus, concentrates cell culture condition media, and isolates exosomes. Ultracentrifugation, microfiltration, size exclusion chromatography, and immunoaffinity may purify exosomes after cell harvest.

Current methods separate exosome vesicles from cells, media, and proteins based on density, size, and surface indicators. There is no standard method for separating exosomes because each method isolates a slightly different population. The target product profile and complexity of the upstream material determine downstream processing [159].

****After purification, exosomes must be cryopreserved in an appropriate container closure system in a storage buffer that ensures vesicle stability. Cryoprotectants are used to minimise osmotic damage and stabilise proteins and cells after freezing at −80 °C [160].

****Exosomes have garnered interest as cell-derived biotherapeutics and drug delivery vehicles, but preservation and storage remain important difficulties that must be overcome to permit their usage in delivery systems. Exosomes can be frozen at −80 °C. Storage impacts depend on isolation source. The paucity of knowledge about exosome storage and stabilisation conditions and storage-mediated effects may limit their clinical use for medication delivery [161].

Even at −80 °C, exosomes are usually unstable, depending on their source. When held at −80 °C for 4 days, exosome shape changed from newly separated. Briefly, new BALF exosomes have a distinct shape and mean diameter. Multilamellar structure development increases BALF exosome diameter by 10% at +4 °C and 25% at −80 °C. Exosomes maintained at different temperatures can also leak protein groups or dissociate pre-exosomal proteins. Storage conditions destabilise BALF exosome morphology, surface characteristics, and protein content [162].

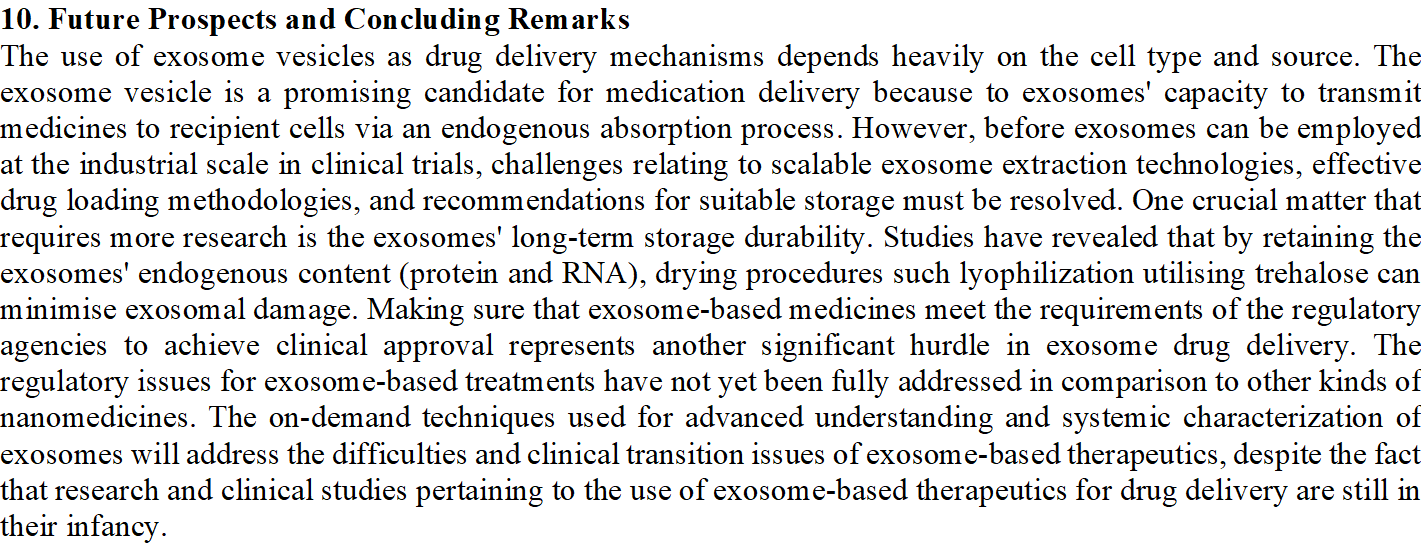
Preserving exosomes during preparation and storage is essential for medication delivery. Maintaining biological activity and repeatability in downstream processing requires various exosome preservation methods in solution and solid state. Exosome delivery systems frequently have issues with particle aggregation during high-speed centrifugation, interactions of highly enriched extracellular vesicle suspensions during storage, and freezing damage. A good preservation approach can increase exosomes' clinical therapeutic potential. Exosomes are stored at −80 °C, but this may not be optimum for shipping and handling. Thus, exosome storage stability requires alternative strategies. Cryopreservation and lyophilization or freeze-drying can preserve exosomes [163]. Exosome functions are preserved by freezing, thawing, and refreezing after use.

Cryopreservation is simple and available, but exosomes cannot be preserved above −20 °C and repetitive freezing and thawing may damage them. Thus, lyophilization may preserve exosomes [164].

Lyophilization preserves exosomal formulations for long-term room-temperature storage. Lyophilization is favoured over freezing because it removes freezable water from exosome contents, making them more stable [165].

Lyophilization reduces physical deterioration of vesicles by preventing phospholipid hydrolysis. It may also stabilise exosome active components. Sugars, especially trehalose, have been utilised as cryo- or lyoprotectants during lyophilization to prevent leakage and preserve membrane integrity. Trehalose is an α-1,1-glycosidic connection between two glucose units. Trehalose narrows exosome particle size distribution and increases particle count per microgram of protein. Trehalose also reduces freeze–thaw vesicle aggregation and preserves lyophilized exosome particle properties. Trehalose reduces vesicle fusion and exosome loss during lyophilization in in vitro electroporation experiments [166].

Intracellular and extracellular cryoprotectants are typically classified as such. Dimethyl sulphoxide, glycerol, and ethylene glycol penetrate cells to prevent ice crystals and membrane disruption. Sucrose, trehalose, and other extracellular sugars function differently [167]. Trehalose replaces water and vitrifies exosomes during lyophilization. The water replacement mechanism replaces water molecules with a stable hydrogen bond between sugars (trehalose) and exosome lipids at the bilayer surface without changing the lipid bilayer structure. Sugars also diminish van der Waals interactions between phospholipid acyl chains and maintain membrane structure after lyophilization [168]. Vitrification involves the immobilisation of molecules (proteins or lipids) by the stabilizer's glassy matrix (trehalose) after water removal. Thus, the glassy matrix, with poor mobility and high viscosity, prevents aggregation or fusion and protects lipid bilayers and protein molecules from ice crystal damage. Trehalose suppresses lipid phase transition conformational alterations [169].

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