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. Better understanding of exosome biology and function, as well as the development of nanotechnologies for the precise separation of well-characterized clinical-grade exosomes and their loading with a variety of therapeutic payloads, are required for the clinical translation of exosomal therapies.

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]

Apoptotic bodies, Microvesicles, and Exosomes are the three categories of EVs based on their intracellular origins. Apoptotic bodies are 50 to 5000 nm in size and include biological components such deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and histone proteins. Apoptotic bodies convey these contents to macrophages during apoptosis, resulting in cell engulfment [2,3]. Other names for microvesicles include ectosomes, shedding vesicles, microparticles, plasma membrane-derived vesicles, and exovesicles. Microvesicles range in size from 50 nm to 1000 nm and are generated by the outward budding and fission of plasma membranes. When microvesicles are created, they transport certain proteins and lipids to a selected destination cell [4]. Exosomes, the final class of EV, vary from microvesicles primarily in their intracellular origin and size (Fig 1). Exosomes are thought to be the most promising as a method for medication delivery to targeted organs due to their nanoscale dimensions, hence they have gained the greatest scientific focus in recent years.

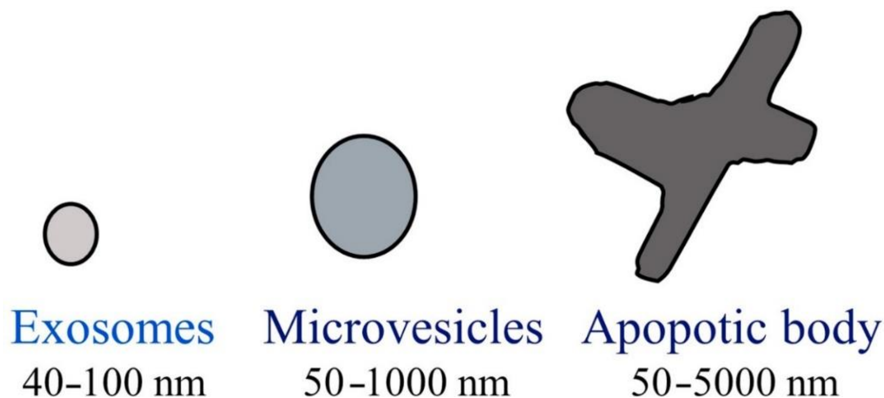

Fig 1. Types of Microvesicles

1. Discovery of Exosomes

Rose Johnstone and her colleagues coined the term "exosome" in 1970. They observed the production of "an intracellular sac filled with small membrane-enclosed structure of nearly uniform size" while dealing with maturing reticulocytes. In contrast to endocytosis, when external molecules are internalised into the membrane-bound structure, they formed intracellular vesicles and discharged contents outside the cell. As a result, these intracellularly produced vesicles were given the name "EXOSOME" [5].

Exosomes develop from endosomes with sizes ranging from 40 to 100 nm [6]. They are secreted by all cell types and are found in the majority of bodily fluids, including blood, saliva, and urine. An exosome is a "nanosphere" with a bilayer membrane that contains lipids and proteins from the parent cell. Transport proteins, heat shock proteins, proteins involved in multi-vesicular body biogenesis (MVB), and tetraspanin are among these proteins.

Exosomes are made up of a variety of lipids, including cholesterol, sphingolipids, phosphoglycerides, ceramides, and saturated fatty acid chains [7]. Exosome composition is important because it serves as a biomarker and indicates how exosomes function in biological processes.

2. Biogenesis of Exosomes

In general, exosome production is divided into three stages:
(1) Formation of endocytic vesicles from plasma membrane,
(2) Inward budding of the endosomal vesicle membrane resulting in MVBs that consist of intraluminal vesicles (ILVs), and
(3) Fusion of these MVBs with the plasma membrane, which releases the vesicular contents, known as exosomes [7] (Fig 2).
In the first stage, endocytic vesicles are formed from the plasma membrane, creating an early endosome, which is then matured into late endosomes. The limiting membrane of these late endosomes undergoes inward budding, in turn forming vesicles inside the lumen. The accumulation of these ILVs inside the late endosomes is termed as MVBs. There are two known pathways to the formation of MVBs. One pathway involves endosomal sorting complexes required for transport (ESCRT) and another pathway is ESCRT independent. These MVBs can either fuse with lysosomes for destruction or fuse with the cell's plasma membrane, releasing ILVs into extracellular space, which are known as exosomes [8].

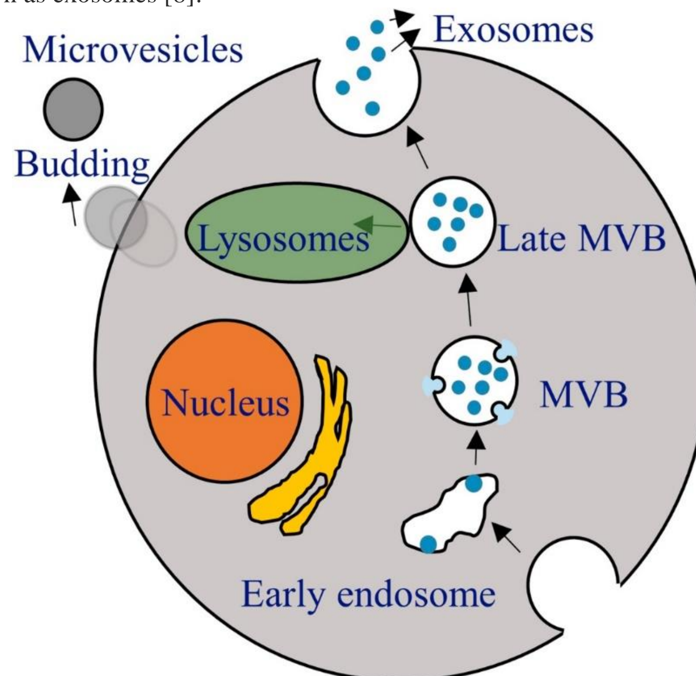

Fig 2. Formation of Exosome and Microvesicle.

Exosome is derived from endosome formed from plasma membrane. As early endosome becomes late endosomes, inward budding occurs and forms multivesicular bodies (MVB) containing numerous intraluminal vesicles (ILV). MVB can either get degraded by lysosomes or fuse with the membrane to release ILV called exosomes. Microvesicles, on the other hand, originate from the budding of the plasma membrane.

The ESCRT is made up of four soluble multi-protein complexes known as ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. The complex is called upon to sort specific proteins into ILVs. ESCRT-0 is in charge of cargo clustering, which is essential for endocytosed receptor ubiquitination [9]. These payloads are proteins that will be integrated into ILVs and then become part of the exosomes that are released. TSG101, an ESCRT-I component, forms a compound with the ubiquinated cargo protein and aids in the activation of the ESCRT-II complex, triggering bud development. This complex subsequently sequesters MVB proteins and recruits the de-ubiquitination enzyme to remove ubiquitin from cargo proteins before sorting them into ILVs. ESCRT-III is deconstructed in the final stage by vacuolar protein sorting-associated protein 4 (VPS4) adenosine triphosphatase (ATPase) [10,11].

Exosome secretion can be inhibited by chemicals that inhibit ESCRT, according to research. Four independent studies, for example, found that inhibiting the HRS member of ESCRT-0 reduced exosome secretion in a variety

of cell types, including Henrietta Lacks cells (HeLa)—a cancer human epithelial cell lineage, mouse dentritic cells, human embryonic kidney cell line 293 (HEK293), and squamous carcinoma cells [12-15]. TSG101 deletion has also been found to inhibit exosome release in tumour cells and immortalised retinal pigment epithelial cells 1 (RPE1) [12,16]. Furthermore, despite ESCRT suppression, multiple investigations have shown the generation of exosomes, indicating the existence of an ESCRT independent mechanism. Lipids, tetraspanins, and heat shock proteins are among the components involved in this separate mechanism [17]. It has been postulated that lipids like as ceramides cause the inward curving of the limiting membrane of MVBs, resulting in the formation of ILVs. Cholesterol and phosphophatidic acid are two more lipids linked to the production of exosomes via ESCRT-independent pathways. Aside from lipids, proteins like tetraspanins have been linked to exosome cargo sorting [18] and the integration of melanosomal proteins into ILVs in an ESCRT-independent way [19].

3. Fundamentals of the Exosomes

3.1. Structure and Composition of Exosomes

Exosomes construct a phospholipid outer envelope, and the inner core carries a group of biologically active molecules. Components of exosomes are proteins, lipids, nucleic acids, and glycoconjugates (Fig 3).

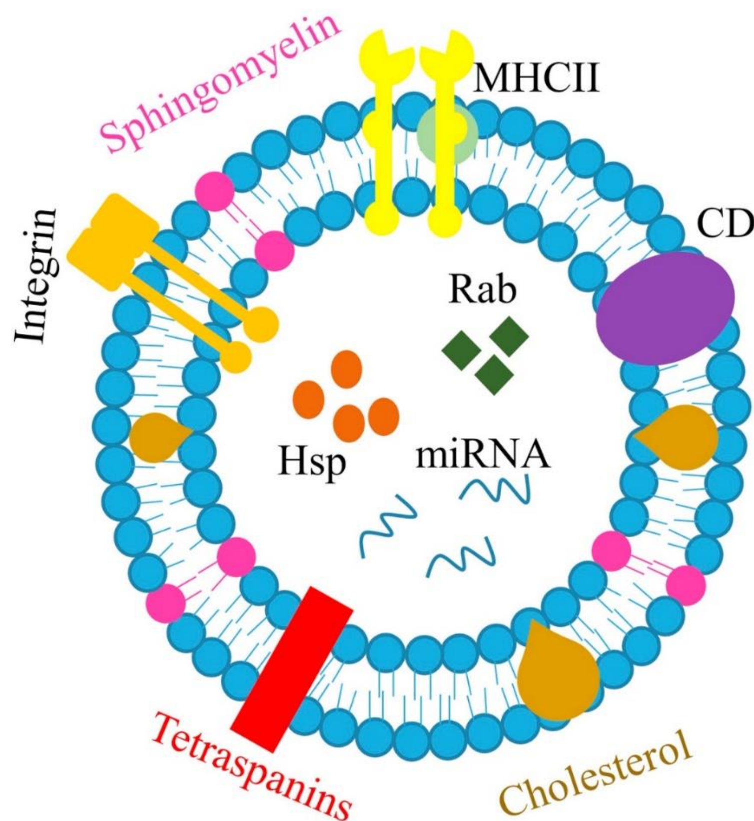

Fig 3. Composition of exosomes.

Exosomes are composed of various types of proteins, such as major histocompatibility complex (MHC)-II, integrin, cluster of differentiation (CD), tetraspanins, heat shock protein (Hsp), Ras-related protein (Rab), etc. Exosomes also contain various types of lipids, such as sphingomyelin and cholesterol. Lastly, exosomes are found to contain nucleic acid, including miRNA, mRNA and non-coding RNAs.

Table 1. Summary and examples of Exosomal compositions

Exosome composition	Example
Proteins	**Heat shock proteins:** Hsp70, Hsp90
	Membrane transport and fusion proteins: GTPase, annexins, flotillin
	Tetraspanins: CD9, CD63, CD81, CD82
	Thrombospondin
	ALIX
	TSG101
Lipids	Sphingomyelin
	Phosphatidylcholine
	Phosphatidylethanolamine
	Phosphatidylserine
	GM3
	Phosphatidylinositol
Nucleic acids	mRNA
	miRNA
	Non-coding RNA

Since exosomes originate from the intracellular component "Endosome", they contain proteins such as heat shock proteins (Hsp70 and Hsp90), membrane transport and fusion proteins (GTPases. Annexins and flotillin), and tetraspanins (CD9, CD63, CD81, and CD82) [24]. Heat shock proteins, annexins, and Rab family proteins are prevalent in exosomes and play important roles in their intracellular assembly and trafficking. Another protein found in exosomes is tetraspanins, a transmembrane protein family. Tetraspanins play a role in cell fusion, motility, cell-cell adhesion, and signalling. However, the function of exosomes is poorly unknown [2]. Integrins, which are adhesion molecules that allow cell attachment to the extracellular matrix, are another common protein present in exosomes. Integrins are crucial in the adhesion of exosomes to their target cells [20]. All of these proteins have been used to identify the presence of exosomes. Exosomes are also related with the proteins thrombospondin, CD55, CD59, lactadherin, ALIX, and TSG10 [21]. These multiple proteins are integrated into exosomes during exosome synthesis and function as cargo for cell-cell communication.

Besides proteins, exosomes are also rich in lipids, with different types of exosomes containing different types of lipids. 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]. Exosomes can also be formed by cholesterol, ceramide, and phosphoglycerides, as well as saturated fatty-acid chains. Exosomes also include nucleic acids such as micro RNA (miRNA), messenger RNA (mRNA), and non-coding RNAs.

3.2. Exosome Sources

Exosomes are separated from biological fluids such as blood, urine, and saliva. [25,26] They can also be obtained from the tumour microenvironment, as tumours produce a greater number of exosomes than normal cells. Exosomes can also be produced by HEK293 cells (human embryonic kidney cells), HeLa cells, and a variety of other cells. Exosomes, in addition to being a source of cancer, are also a source of other important chemicals. The primary sources of vaccine development are DCs, NKs, and exosomes secreted by tumour cells. Exosome-based cancer vaccines derived predominantly from mesenchymal stem cells and macrophages have also been reported. [27,28]

3.3 Isolation and characterization of exosomes

Differential centrifugation, filtration, size-exclusion chromatography, and polymer precipitation are used to isolate exosomes (Fig. 4). An immunomagnetic isolation approach targeting exosomal markers can be used to extract ultrapure exosomes or isolate a possible subpopulation of exosomes.

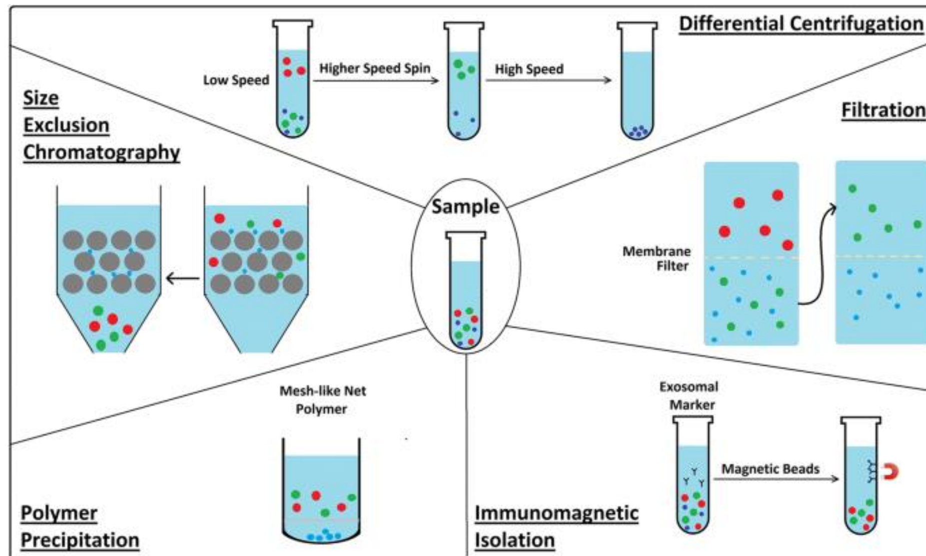

Fig 4: Schematic diagram of exosome isolation

Differential centrifugation

Differential centrifugation is the most extensively used method for isolating exosomes from biological fluids; it is recognised as a consistent approach for isolating exosomes from biological fluids. The procedure includes three steps:

(1) a low-speed centrifugation to remove cells and apoptotic debris,
(2) a higher-speed spin to remove bigger vesicles, and
(3) a high-speed centrifugation to precipitate exosomes.

When viscous biological fluids such as plasma and serum are utilised for analysis, the method's efficiency is reduced. Because centrifuging speed and time affect the amount of EVs collected, these parameters must be optimised for each rotor type [29].

Filtration

To separate cells and big EVs in biological samples, utilise commercial membrane filters or polycarbonate. Ultracentrifugation is frequently employed in conjunction with filtration techniques to separate exosomes from proteins. Membranes are utilised to sift cells and big EVs. A number of research teams have looked into commercial ultrafiltration as a way to separate exosomes from protein impurities in order to do away with the necessity for ultracentrifugation. Using matrices with set molecular weight or size exclusion criteria, exosomes can be isolated from other soluble proteins and aggregates. Based on a molecular weight larger than 2 million Daltons and a diameter less than 200 nm, these vesicles can be extracted selectively. This makes it possible to separate exosomes from smaller aggregates and soluble components [30].

Size-exclusion chromatography

In size-exclusion chromatography (SEC), often referred to as molecular sieve chromatography, various substances are separated based on their hydrodynamic volume, which is determined by how well they enter the pores of the stationary phase. Two fundamental types of size exclusion chromatography exist. The procedure is known as gel permeation chromatography (GPC) when carried out using organic solvents. GPC's primary area of use is polymer analysis. Gel filtration is the term used to describe size-exclusion chromatography that is carried out with aqueous solvents. This method has three drawbacks:

(1) the chromatography column is susceptible to contamination, so aseptic working conditions must be maintained, especially if the isolated EVs are intended for therapeutic use;
(2) numerous fractions must be collected and analysed to ensure complete separation of EV subtypes and contaminating proteins; and
(3) in contrast to the separation protocol's simplicity and time effectiveness, post-isolation amplification is required.[31]

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].

Immunomagnetic isolation

The exosomes are separated from other substances using the immunoisolation or immunoaffinity approach, which employs magnetic beads covered with antibodies to recognise specific proteins on the lipid bilayer membrane [33]. According to reports, biomarkers including CD34, CD63, and CD326 are frequently employed to identify human exosomes, tumour exosomes, and acute myeloid leukaemia blasts, respectively [34]. Tetraspanin proteins and exosome surface indicators, which are thought to be determining factors for the immunoisolation technique, can be utilised to isolate the exosomes using an immunoaffinity isolation kit (microplate-based enzyme linked immunosorbent assay, or ELISA) [35]. The immunoisolation approach is better than ultracentrifugation in capturing a tiny amount of plasma with excellent specificity. As a result, it is frequently utilised to further separate the particular exosomes that have already been isolated using conventional methods. However, this method can be used to isolate exosomes that are particular to the desired biomarkers [36,37].

Microfluidics-Derived Chip Isolation Methods

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.

3.4 Biological functions of Exosomes

- Many cells produce exosomes into biological fluids like synovial fluid, breast milk, blood, urine, saliva, amniotic liquid, and in-blood serum, suggesting they are important for intercellular communication and physiological reactions.
- Exosomes first remove unneeded proteins during cell maturation [38,39].
- Exosome functions vary by origin. Exosomes from antigen presenting cells express major histocompatibility complex (MHC) class I and II molecules, which activate CD8+ and CD4+ T-cells to induce particular immunological responses. [40,41].
- By carrying prostaglandins, exosomes secreted by platelets are involved in the inflammatory response.
- Mast cells produce exosomes carrying mRNA and short RNA, which are transported to selected recipient cells and translated into the recipient cell. [42].
- Glioblastoma cells produce exosomes with mRNA, miRNA, and angiogentic proteins to microvascular endothelial cells, encouraging angiogenesisient cell growth. [43]
- Facilitate cell-to-cell communication in a target-specific manner throughout the body.
- Involved in maturation of erythrocytes, the elimination of unnecessary proteins and RNA [44], antigen presentation in immune responses [45], coagulation, inflammation, and angiogenesis.
- It has been discovered that exosomes can transmit horizontal miRNA. The exosome's ability to carry cargo facilitates these activities, and each exosome's composition is determined by the origin of the parental cell from which it was formed.
- Many diseases are linked to exosomes. Exosomes carry mRNA and protein, like mutant Kirsten rat sarcoma (kRAS) viral oncogene protein and c-Met oncoprotein, to distant regions, encouraging angiogenesis, thrombosis, and tumour cell proliferation. [46-48].
- Exosomes carry misfolded proteins from unhealthy neurons to surrounding cells, spreading neurodegenerative diseases like Parkinson's disease (PD). Other disorders, such as myocardial infarction and HIV, include exosomes.[49]

- Exosomes detect prostate, breast, and ovarian malignancies. They contain and express infectious RNA and proteins, which helps diagnose infectious disorders and detect active and latent intracellular infection. Exosomes provide growth factors, proteins, miRNA, mRNA, non-coding RNA, and lipids to regenerate tissue. In a myocardial infarction and kidney injury model, stem cell and endothelial progenitor cell exosomes regenerated heart tissue and neovascularization. [50-52]

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.

An essential first step in exosome and T cell contact is cellular identification of the exosome via adhesion. In order to permit the high avidity binding of lymphocyte to the integrin-bound exosomes, adhesion by exosomes necessitates the conformational change of integrins from a low to a high affinity status, which facilitates oligomerization of integrins and coupling with cytoskeletal components.

According to molecular profiling and proteomic study, target cell specificity for exosomes is determined by a combination of antigen and MHC class I and II molecules in the case of cellular identification by antigen recognition. Exosome MHC expression is reliant on the parent cell's expression of these molecules. [12].

3.6 Exosome uptake

Exosome uptake can be described as a three-step process that involves receptor interaction, membrane fusion, and endocytosis/phagocytosis to deliver exosome signals to the destination cell (Fig. 5). Numerous studies have also suggested that internalisation, which is influenced by cell type and exosomal surface proteins, is the main way of exosome uptake. Exosome uptake by a recipient cell is cell-specific, and exosome targeting and adherence depend on the interaction of certain cells' surface chemicals with exosomes [13].

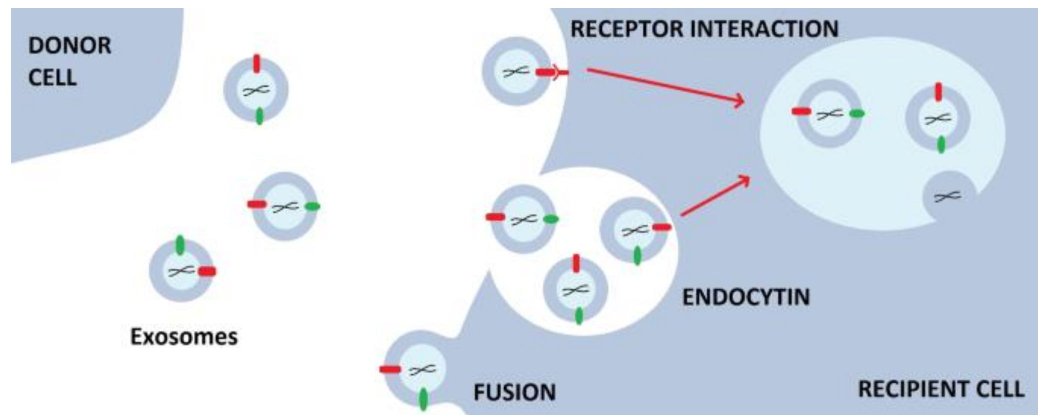

Fig 5: Exosome uptake

3.7 Derived Exosomes

Exosomes have different roles depending on their progenitor cell. A significant amount of research has been carried out in an attempt to identify the roles of varied derived exosomes.

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.

The purpose of recent research has been to understand the function of exosomes produced by macrophages. The findings indicate that the connection between the integrins ICAM-1 and LFA-1 was responsible for the unbiblical vein endothelial cell's uptake of macrophage exosomes, and that the c-type lectin receptor mediated this process in hCMEC/D3 cells. The study also discovered that macrophage exosomes can cross the blood-brain barrier to reach the brain parenchyma in healthy mice; they can even enter the brain on their own, without the assistance of immune cells that have infiltrated the brain, and the increased accumulation in the inflamed brain was caused by improved exosome-brain endothelium interaction.[57]

Rhabdomyosarcoma (RMS)-derived exosomes

Rhabdomyosarcoma (RMS), the most prevalent soft tissue sarcoma (STS) in children and adolescents, has skeletal muscle lineage [58]. This affects youngsters, median-age, and elderly patients. RMS is a "small round blue tumour cell" that infiltrates bone marrow (BM) and may be diagnosed as acute leukaemia on BM smears. RMS has two histological subtypes: embryonal and alveolar. ARMS is more aggressive than ERMS and has a worse prognosis [59].

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].

Metastatic cancer cell-derived exosomes

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 (MM) cell-derived exosomes

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].

Pancreatic cancer cell (PCC)-derived exosomes

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].

Bronchial fibroblast-derived exosomes

Airway inflammation is caused by fibroblasts producing cytokines and adhesion molecules and infiltrating and activating eosinophils and other leukocytes. They are the most abundant cell type in the lung interstitium and can be transformed into the myofibroblast phenotype, which expresses contractile proteins and increases collagen deposition, regulated by cytokines, growth factors, and matrix components [73,74].

Haj-Saleem et al. [75] examined the role of bronchial fibroblast-derived exosomes. Bronchial epithelial cells absorb exosomes released by bronchial fibroblasts. Exosomes from severe asthmatic fibroblasts had reduced TGF-

b2 levels and enhanced epithelial cell proliferation in both healthy and severe asthmatics. Overexpression of TGF-b2 in severe asthmatics' fibroblasts increased exosome TGF-b2, reducing epithelial cell proliferation, while decrease increased it.

In severe asthma, bronchial fibroblast-derived exosomes promote airway epithelial proliferation. Exosomes from severe eosinophilic asthmatics' fibroblasts modulate epithelial cells to rebuild airways [75].

Mesenchymal stem cell (MSC)-derived exosomes
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].

4. Exosome as a therapeutic delivery system

Liposomes and polymeric nanoparticles are the most common drug delivery methods for anticancer, antifungal, and analgesic compounds. However, biocompatibility, greater stability, long-term safety, and ability to escape the host immune system with extended systemic circulation capability and stability remain key problems [81,82].

Exosomes have been developed as nanoscale delivery methods for years. Exosomes are attractive nanocarrier drug delivery systems because of their biocompatiblity and biodegradability, low toxicity, fusogenic properties, low-uptake machinery, high target cell specificity, and small size. Exosomes also accumulate more in tumour tissues than normal tissue. Exosomes can be anchored with tumor-targeting ligands like proteins, peptides, or antibodies to transport drugs more selectively [83,84]. Table 2 illustrates exosome therapeutic delivery in many clinical situations.

Table 2: Examples of Exosomes as Drug Delivery Systems

Cargo Type	Origin of Exosomes	Disease Type	Isolation or Purification Method	Drug Loading Method	Outcome	Reference
Proteins						
Signal regulatory protein α	Human embryonic kidney293T cells	Cancer	Centrifugation	Transfection	Enhanced phagocytosis of tumor cells	[85]
Survivin-T34A	Melanoma cell lines	Pancreatic cancer	Centrifugation	NA	Apoptotic death of cells	[86]
Antiepidermal growth factor receptor	Mouse neuroblastoma	Epidermoid carcinoma	Ultrafiltration/size exclusion liquid chromatography	NA	Target specificity	[87]
20S proteasome	Mesenchymal stem cells	Mouse myocardium	Tangential flow filtration	NA	Reduction in myocardial infraction	[88]
Genetic Substances						
miRNA	Glioblastoma cells	Glioblastoma tumor	Differential centrifugation	Transfection	Providing diagnostic information	[89]
miRNA	Human cord blood endothelial colony-forming cells	Ischemic kidney injury	Centrifugation	Transfection	Protected kidney function and reduced kidney injury	[90]
Spherical nucleic acids	PC-3 cells	Prostate cancer	Centrifugation	Naturally encased	3000-fold-enhanced knockdown of miR-21	[91]

siRNA	Human embryonic kidneycells (HEK293)	Breast cancer	Sequential centrifugation	Electroporation	TPD52 gene expression was downregulated up to 70% compared with non-targeted exosomes	[92]
Small molecules						
Paclitaxel	Prostate cancer cell lines (PC-3 and LNCaP)	Autologous prostate cancer	Differential centrifugation	Co-incubation	Enhanced drug cytotoxicity to cancer cells	[93]
Doxorubicin	Immature mouse dendritic cells transfected with the vector-expressing iRGD-Lamp2b fusion proteins	Breast cancer	Centrifugation and ultrafiltration	Electroporation	Specific drug delivery to the tumor site andinhibited tumor growth	[94]
Curcumin	Tumor cells (GL26-Luc, BV2, 3T3L1, 4T1, CT26, A20, and EL-4)	Brain tumor and autoimmune encephalitis	Sucrose gradient centrifugation	Direct mixing	Inhibited brain inflammation and delayed brain tumor growth	[95]
Dopamine	Kunming mouse blood	Parkinson's disease	Ultracentrifugation	Co-incubation	Enhanced therapeutic effect due to brain specific drug delivery	[96]

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].

Exosomes naturally include membrane-associated protein ligands. These bioactive ligands cluster into microdomains on exosomes, creating a natural membrane environment for biomacromolecules that maintains stability and bioactivity, improving membrane protein therapeutics [85].

Heat shock proteins, annexins, and Rab family proteins, which are prevalent in exosomes, are mainly engaged in exosome trafficking and intercellular assembly and may not benefit medication delivery. Other protein therapies may deliver exosomes [98].

Survivin-T34A, a dominant-negative mutant of the apoptotic protein Survivin, was successfully inserted into exosomes from melanoma cell lines to induce apoptosis [99]. Kooijmans et al. attached glycosylphosphatidylinositol-anchored antiepidermal growth factor receptor nanobodies to exosome vesicles to increase exosome-tumor cell interactions [87].

GALA-modified exosomes were made by mixing dendritic cell-derived SAV (a protein that binds biotin with high affinity) and LA (an exosome-tropic protein) with pH-sensitive GALA peptides. These modified exosomes affect tumour cell intercellular trafficking and antigen presentation [100]. Alphagalactosylceramide or ovalbumin-loaded exosomes may also produce an adaptive immunological response without inducing natural killer T-cell anergy [101].

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.

Cytosolic proteins like tubulin and actin, protein kinases, Annexin and Rab family proteins, tetraspanins (CD9, CD63, CD81), heat shock proteins (HSP 70, HSP 90), and transmembrane proteins are found in all exosomes [103]. Antigen-presenting cell exosomes mimic CD8+ and CD4+ T cells by carrying tetraspanin CD86 and major histocompatibility complex components I and II. Pathogen-associated molecular patterns on the exosome membrane may activate immune cells [104]. New vaccine formulations may use exosomes to boost and modify immune responses. Nanoscale exosomal vesicles can activate granulocytes or NK cells and interact with CD8+, CD4+, and B cells to produce antigen-specific immune responses [105].

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. Kim et al. [110] investigated genetically engineered exosomes that express a targeting ligand to increase exosome delivery to a target organ and reduce systemic toxicity. Briefly, cardiac-targeting peptide, a targeting ligand generated from genetically modified exosomes, was tested for tissue and heart cell delivery in vitro and in vivo. Exosomes obtained from HEK293 cells by differential centrifugation were genetically edited by fusing cardiac-targeting peptide (CTP)–Lamp2b on the membrane (CTP–Exo), while CTL–Exos were used as controls. H9C2 rat cardiomyocytes delivered more CTP–Exo than CTL–Exo in the in vitro investigation. CTP–Exo delivered 15% better than CTL–Exo in vivo, indicating that genetic alteration of exosomes with targeting peptides can be used to treat heart disorders due to improved delivery and less systemic toxicity. Exosomes containing catalase improved Parkinson's disease in patients after crossing the BBB. Catalase distribution over the BBB is difficult, however putting it into the exosome carrier system may help treat Parkinson's disease.

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)

Eukaryotic cells have non-coding RNA called miRNA with non-protein nucleotides. MiRNAs influence post-transcriptional gene expression by binding to complementary regions on targeted mRNA [117]. Exosomes naturally carry miRNA, therefore they can be used to deliver miRNA to particular cells. Ohno et al. [119] used exosomes to deliver miRNA targeting EGFR in breast cancer cells. Epithelial tumours expressed high levels of EGFR, suggesting that EGFR ligand could be cancer therapeutic targets [118]. MiRNAs like let-7a may prevent cancer growth by lowering RAS and HMGA2 expression. Epidermal growth factor (EGF) and EGFR-specific peptide (GE11) were added to exosomes carrying let-7a to transport it to EGFR-expressing cancer tissue60. Transfected HEK-293 cells with a pDisplay vector expressing GE11 or EGF produced GE11- and EPF-positive exosomes. Differential centrifugation separated exosomes from cloned cells. Anti-hemagglutinin and western blot examination assessed exosome GE11 or EGF expression. Fluorescence-activated cell sorting and anti-Myc-tag antibodies confirmed GE11 or EGF on exosomes. GE11- or EGF-positive exosomes were examined for EGFR binding in HCC70, HCC1954, and MCF-7 breast cancer cell lines. PKH67 dye was used to demonstrate exosomes entering recipient cells. EGFR-dependent exosome uptake was confirmed by several tests. The modified exosomes entered recipient cells. Cell proliferation experiments were also used to determine if GE11- or EGF-positive exosomes altered cell growth. GE11-positive exosomes did not promote EGFR signalling like EGF, therefore they may be better.

Lethal-7 gene (let-7a) was lipofected into GE11 exosomes in vivo. Then, tumor-bearing animals received intravenous let-7a-containing GE11 exosomes. Since previous research demonstrated let-7a suppressed tumour growth by reducing RAS and HMGA2 expression, real-time reverse transcription-PCR, immunoblotting, and immunostaining were performed on injected tumor-bearing animals. GE11-exosome-delivered let-7a substantially suppressed HMGA2 expression in cancer cells. This suggests that exosomes can deliver drugs to target cells [119].

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.

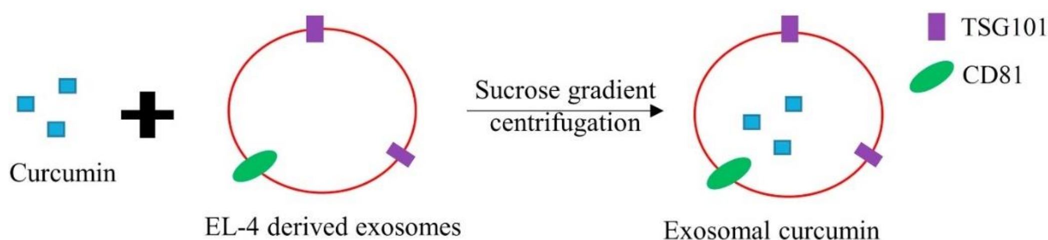

Fig 6. Formation of Exosomal curcumin

Curcumin was incorporated into murine tumor cell line (EL-4), derived exosomes and isolated using sucrose gradient centrifugation

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).

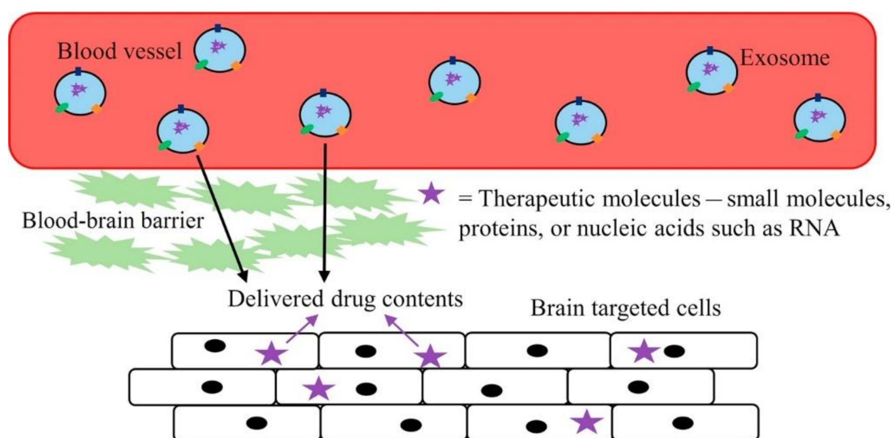

Fig 7. Ability of exosome and its drug contents to cross the blood–brain barrier.

Encapsulating anticancer medicines like paclitaxel and doxorubicin into exosomes has shown their potential for brain delivery across the BBB and explained their transport processes in zebrafish [124]. Using Invitrogen® total exosome RNA and a protein separation kit, exosomes from gliobastoma astrocytoma U-87 MG, endothelium bEND.3, neuroectodermal tumour PFSK-1, and glioblastoma A-172 were extracted. Mixing and incubation loaded exosomes with rhodamine 123, paclitaxel, or doxorubicin. The isolated exosomes, rhodamine 123-containing exosomes, and U-87 MG and bEND.3 cell exosomes were characterised. bEND.3-derived exosomes loaded with rhodamine 123, doxorubicin, or paclitaxel were injected into zebrafish embryos to test their BBB-crossing capacity. Rhodamine 123 fluorescence was measured in brain tissue after the tests. The zebrafish embryos' brains revealed drug dispersion, showing exosomes can transfer medications across the BBB. Zebrafish were used to create a primary brain cancer model to compare exosome-loaded anticancer medicines. In the zebrafish brain model, exosome-loaded doxorubicin was more effective than doxorubicin alone. The study indicated exosomes could carry small molecule medications across the BBB to treat brain tumours and neurological diseases.

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.

Exosomes are employed for transporting pharmaceuticals over the BBB in addition to enhancing the characteristics of small-molecule pharmaceuticals. The majority of the potent central nervous system medications have not been successful in clinical trials because the majority of these compounds cannot cross the BBB due to the issues with the permeability of small-molecule pharmaceuticals across the BBB. Exosomes, which are the body's own cells, are used as delivery vehicles to customise medications so they may pass across the BBB, improving drug transport to the brain by reducing mononuclear phagocyte system drug clearance [12].

5. Exosomes Drug Loading Techniques

The exosome vesicle's lipid bilayer wall prevents blood circulation cargo breakdown. The lipid bilayer membrane and endogenous composition of exosomes make medication loading difficult. Active and passive drug loading strategies can sort exosomes. Active loading involves incubating the medication with isolated exosomes. Passive drug loading, or preloading, involves drug-sorted exosomes from pretreated donor or source cells. This approach does not involve exosome vesicle medication addition. Due to its active pumping mechanisms, active loading can achieve a greater drug/vesicle ratio. Postloading works best for hydrophobic drugs [127]. Fig 8 shows exosome drug loading methods, while Table 3 lists their pros and cons.

Table 3: Pros and Cons of different exosome drug loading approaches

Drug Loading Approach	Mechanism	Advantages	Disadvantages
Passive Loading			
Incubation of exosomes and free drugs.	Diffusion of cargo into a cell or exosomal membrane.	Simple operation. Does not compromise the membrane integrity.	Loading efficiency. Drugs may cause cytotoxicity to the donor cells.
Incubation of the donor cells with free drugs.			
Active Loading			
Sonication	Creation of micropores for diffusion by mechanical shear force.	Higher loading capacity than the simple incubation method.	Sonication-induced membrane degradation prevents large-scale use. Affects exosome integrity and cargo aggregation.
Extrusion	Membrane recombination.	High cargo loading efficiency. Repeated extrusion provides a homogeneous blend of exosomes with cargoes.	Recombination of exosomal surface structure may expose exosomes to immune cells such mononuclear phagocytes.
Freeze–thaw cycles	Membrane fusion.	Simple and effective strategy to load various cargoes (drugs, proteins, and peptides) into exosomes directly.	Repeated freeze–thaw may degrade proteins and aggregate exosomes. Lower drug loading efficiency
Electroporation	Creation of micropores for diffusion by the electric field.	High loading efficiency	Loading and cargo aggregation are significant concerns.
Incubation with membrane permeabilizers	Dissolves membrane molecules (cholesterol), create pores on the exosomal surface.	Higher loading capacity as compared with the simple incubation method	*In vivo*, saponin is hemolytic, limiting its drug loading concentration (toxicity). Saponin may require additional purification.

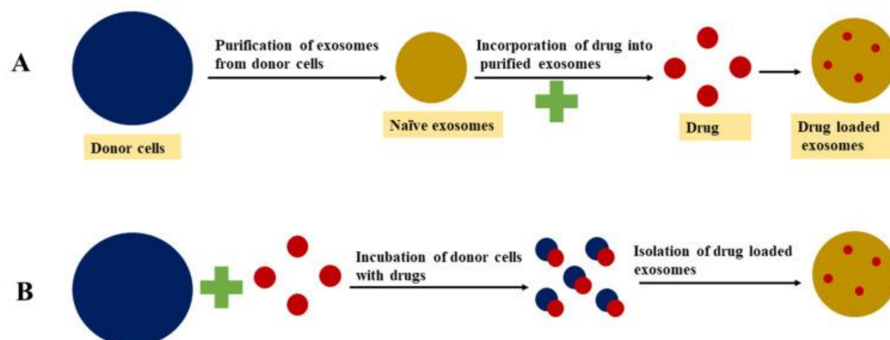

Fig 8: Exosomal drug loading approaches: (A) Postloading approach; (B) Preloading approach

5.1. Passive Loading Approach

5.1.1. Incubation of Drugs with Exosomes

In this passive drug loading procedure, drug and exosomes are incubated together and drug diffuses into exosomes along the concentration gradient. Hydrophobic drugs interact with the vesicle's lipid bilayer membrane, which affects drug loading efficiency [128].

5.1.2. Incubation of Drugs with Donor Cells
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].

5.2. Active Drug Loading Approaches
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].

5.2.1. Sonication
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].

5.2.2. Extrusion
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].

5.2.3. Freeze–Thaw Cycles
The freeze-thaw method for drug loading is incubating exosomes with a targeted drug at ambient temperature for a predetermined amount of time, followed by a quick freeze at 80 °C or in liquid nitrogen. The mixture is then allowed to defrost at room temperature. Freeze-thaw cycles are conducted for at least three cycles to improve drug encapsulation. Compared to extrusion or sonication techniques, this method has a lesser capacity for drug loading. Additionally, this method may encourage exosome aggregation, resulting in a wide range of sizes for the drug-loaded exosomes [128].

5.2.4. Electroporation
Electroporation disrupts the phospholipid bilayer of exosomes, creating pores that allow drug molecules to enter the lumen [131]. After electroporation, drug molecules diffuse through the exosome lipid bilayer membrane pores, restoring membrane integrity. Loading big molecules like nucleotides (siRNA or miRNA) into exosomes is common using this strategy. RNA aggregation and exosome instability limit electroporation loading. This method increases exosome RNA and hydrophilic small molecule loading [127].

5.2.5. Incubation with Membrane Permeabilizers
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.

6. Exosomes Administration Routes
Several delivery methods, including intravenous and intratumoral ones that could result in systemic dispersion of exosomes, have been tested to deliver the therapeutic agent or cargo-loaded exosomes to the target tissue or organ. The tissue distribution of exosome-loaded drugs can be affected by the route of delivery in vivo. Table 4 lists the benefits and diseases that exosomes supplied by various ways target.

Table 4: Targeted diseases of exosomes administered via different routes [164-168].

Routes of Administration	Targeted Disease

Intravenous	Stroke, Parkinson's disease, Traumatic brain injury, Acute kidney injury, Antitumor therapies (prostate and breast cancer).
Intraperitoneal	Bronchopulmonary dysplasia Autoimmune type 1 diabetes
Oral	Facilitates resolution of colitis, Arthritis
Intranasal	Brain parenchyma Brain cancer Encephalitis (inflammation of the brain), Parkinson's disease therapy
Intratumoral	Glioblastoma multiforme antitumor therapies

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

Compared to other systemic administration methods, exosome delivery via the intraperitoneal route enables the loading of higher exosome dosages. Due to the size of the peritoneal cavity, exosomes injected through this route, however, rapidly diluted and expanded to distant regions [136].

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.

6.5. Intranasal Administration

The route of intranasal administration is more efficient, especially in avoiding the difficulties associated with transporting medications across the blood-brain barrier (BBB). By bypassing intestinal and hepatic metabolism, the intranasal approach reduces exosome loss and keeps exosome vesicles in the brain tissue [131]. According to studies, intranasal injection of exosomes containing curcumin and cucurbitacin caused their distribution to the mouse brain to occur quickly. Through this pathway, cucurbitacin-loaded exosomes accelerated tumour apoptosis and inhibited the development of illness in mouse models. The number of microglial cells was significantly decreased by curcumin-loaded exosomes. Exosomes have also been effectively employed to convey therapeutic cargo to prevent brain tumours and inflammation through intranasal administration [138].

7. 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.

Table 5: Methods, advantages, and limitations of exosome characterization techniques

Quantification Methods	Purpose	Advantages	Limitations
Dynamic light scattering	Exosomes size distribution.	Monodisperse systems are measured at 10 nm. Preserving samples for downstream analysis without pretreatment.	Not appropriate for complex exosome samples with vast size ranges Difficult to differentiate contaminated proteins. Inaccurate with polydispersed and heterogeneous samples.
Nanoparticle tracking analysis Technology	Measurement of size and concentration of exosomes.	Better resolution than flow cytometer. Real-time exosome detection is possible.	Camera and detection thresholds affect exosome quantification.
Atomic force microscopy	Detection of exosomal morphology.	Small sample, no fixing or staining.	Exosomes may change size and shape during mica dehydration.
SEM and TEM	Detection of exosomal morphology.	SEM can directly detect surface structure, while TEM can observe exosome interior structure and particle size distribution.	TEM requires more sample preparation than SEM, making it unsuitable for quick assessment of many samples.
Flow cytometry	Detection of biomarkers of exosomes.	Qualitative and quantitative characterization of exosomes.	The detection limit is 400 nm, several vesicles can be detected as one event, Exosome particle size cannot be quantified, and proteins or antibody clumps limit its application.
ELISA	Exosome protein quantification.	Can quantitatively and qualitatively analyse marker proteins.	Time-consuming, may detect non-exosomal marker proteins,

			complex, less repeatable.
Western blot	Exosome marker protein quantification.	**E**xosomes from cell culture media are simple to analyse and are the standard technique for qualitative and quantitative detection of marker proteins.	**C**annot detect exosomal marker proteins in biological fluid because parental cell type affects their detection. **P**rovides general exosome concentration and size statistics.

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].

7.2. Dynamic Light Scattering (DLS)

DLS, also known as Photon correlation spectroscopy uses Brownian particle motions to create time-dependent scattering intensity variations. DLS is best for measuring monodisperse suspensions. This technique can determine exosome vesicle size when large vesicles are present in the suspension, even in low quantities, which may make it difficult to detect small particles [146]. It does not provide information about the source or biochemical data of the exosomes. DLS has some exosome characterisation limitations: This technique requires a high sample (particle) concentration, which may be difficult to prepare for exosomes; the presence of larger particles in the sample masks the exosome population due to the intensity distribution; this technique cannot accurately determine particle concentration; and the low scattering properties of exosomes can make measurements inaccurate [144].

7.3. Flow Cytometry

Flow cytometry is a technique that passes individual cells through a laser beam at a specific wavelength and detects the emitted fluorescence or scattered light. It can measure the size and structure of the exosome, characterise its surface proteins, and determine the cellular origin of a single exosome vesicle [141]. However, conventional flow cytometry has a detection limit of 200–500 nm, which limits its use.

7.4. Nanoparticle Tracking Analysis (NTA)

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].

7.5. Tunable Resistance Pulse Sensing (TRPS)

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].

7.6.2. Western Blotting

Exosome target proteins are usually detected by Western blotting. Purified exosome samples are treated with buffered lysis solution containing denaturants or protease inhibitors, then separated by dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a membrane for immunoblotting of specific protein targets. This method measures protein sizes. However, the lengthy preparation and processing durations are its main downsides. ELISA may be scaled up for high-throughput experiments, while Western blotting has similar detection limitations [152].

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**.**

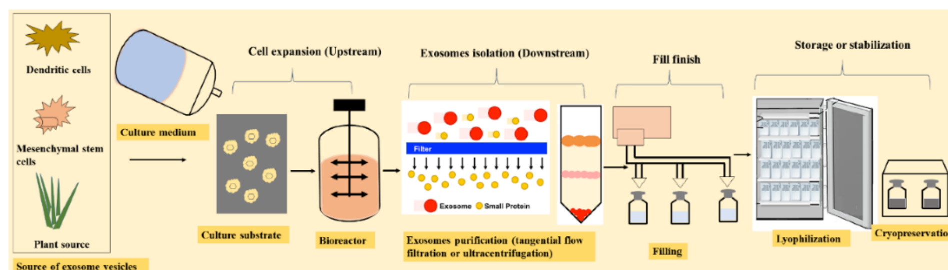

Fig 9: Schematic representation of the manufacturing scheme (upstream and downstream processing) for exosome-based therapeutics.

8.1. Upstream Processing

Cell source and cell culture media are the most crucial exosome manufacturing ingredients. Exosomes are produced using mesenchymal stem cells, dendritic cells, HEK293 cells, and 293T cells in cell culture conditions. MSC exosomes are used to treat many illnesses. Adipose, bone marrow, and umbilical cord tissues can yield MSCs [153]. To choose the best cell source for a therapeutic efficacy and indication, early product development must evaluate different cell types.

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].

8.3. Fill Finish

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].

9. Stability and Stabilization Methods

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].

10. Future Prospects and Concluding Remarks

The use of exosome vesicles as drug delivery mechanisms depends heavily on the cell type and source. The exosome vesicle is a promising candidate for medication delivery because to exosomes' capacity to transmit medicines to recipient cells via an endogenous absorption process. However, before exosomes can be employed at the industrial scale in clinical trials, challenges relating to scalable exosome extraction technologies, effective drug loading methodologies, and recommendations for suitable storage must be resolved. One crucial matter that requires more research is the exosomes' long-term storage durability. Studies have revealed that by retaining the exosomes' endogenous content (protein and RNA), drying procedures such lyophilization utilising trehalose can minimise exosomal damage. Making sure that exosome-based medicines meet the requirements of the regulatory agencies to achieve clinical approval represents another significant hurdle in exosome drug delivery. The regulatory issues for exosome-based treatments have not yet been fully addressed in comparison to other kinds of nanomedicines. The on-demand techniques used for advanced understanding and systemic characterization of exosomes will address the difficulties and clinical transition issues of exosome-based therapeutics, despite the fact that research and clinical studies pertaining to the use of exosome-based therapeutics for drug delivery are still in their infancy.

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