WWW.IJRPR.COM

International Journal of Research Publication and Reviews

Journal homepage: www.ijrpr.com ISSN 2582-7421

A Review on Exosomes: Isolation Techniques, Drug Loading Techniques, Application and Future Perspective.

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Abstract:

Background: Exosomes are nano-sized multicellular vesicles secreted by cells that carry nucleic acids, proteins, lipids, and other bioactive compounds to assist in physiological and pathological processes in the body. when compared to manufactured carriers like as liposomes and nanoparticles, exosomes endogeneity and heterogeneity give them extensive and distinct advantages in disease diagnosis and therapy.

Main body of the abstract: However, exosomes storage stability, low production, low purity, and poor targeting limit their clinical application as a result, further research is necessary to optimise the problems and ease future functional studies of exosomes. By examining a numbers of literatures, the origin, classification, preparation and characterization, storage stability, and applications of exosome delivery systems are summarised and discussed in this work. exosomes isolated and purified using a variety of methods. Drugs can also be loaded onto exosomes to have therapeutic effects.

Short conclusion: The key aim is to increase the production and purity of exosomes, which has traditionally been the barrier restricting its transformation application. Exosome loading capacity, as well as strategies for improving targeting, must be adjusted and improved.

Keyword: exosomes, isolation, loading, drug delivery, multicellular vesicles.

1. Background:

Exosomes are multicellular vesicles generated by all types of cells. They carry nucleic acids, proteins, lipids, and metabolites. They influence several aspects of cell biology and act as both short- and long-range intercellular communication mediators in illness [1]. Exosomes are biological nanoscale spherical lipid bilayer vesicles secreted by cells with a diameter of about 40-100nm and float at a density of 1.13-1.19g ml^{-1} in a sucrose density gradient solution[2-6]. exosomes based carrier that are naturally derived from the cells are now receiving growing attention from scientific community [7]. Due to the following factors, exosomes have been considered as promising natural carriers for drug loading and delivery. exosomes have the ability to pass through various biological and physical barriers in our bodies, such as the Blood brain barrier (BBB) [7]. Exosomes, which are non-immunogenic nano-scale vesicles, are also able to reduce the drug clearance by the mononuclear phagocyte system (MPS) and protect their cargo from enzymatic breakdown [8]. exosomes are naturally generated vesicles that are less toxic than synthetic nanoparticles[9]. Exosomes can be administrated intravenously, intraperitoneal, and intracranial medication delivery, indicating their high degree of flexibility and compatibility [10]. Physical, chemical, and biological methods can be used to selectively load desired cargos on exosomes to achieve a range of therapeutic effects [7].

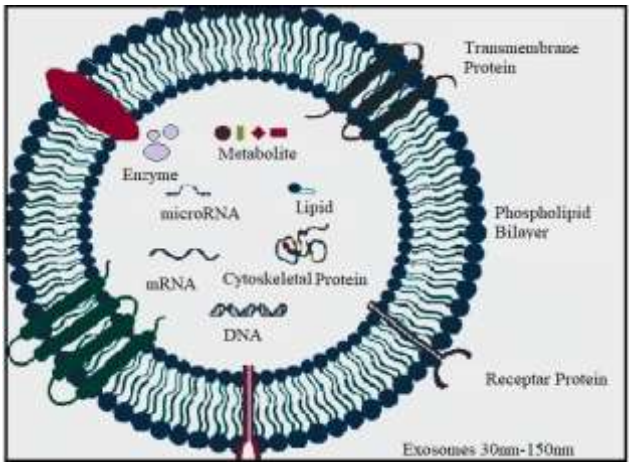

Fig.1 Structure of exosomes

Examples of Exosomes Used as Drug Delivery Carrier:

Donor cell origin	Therapeutic agents	Loading mechanisms	Targeting sites
Immature DC cells (Dendritic cell)	siRNA	Electroporation	Mouse brain [18]
HEK293T (Human embryonic kidney cell)	Suicide mRNA CD-UPRT-EGFP	Pre-transfected parent cells	Schwannoma tumours[19]
Immature DC	Dox	Electroporation	Breast cancer [20]
HEK293 (Human embryonic kidney cell)	Let-7a miRNA	Transfection	Breast cancer [21]
EL-4, MDA, 4T-1	Curcumin	Sucrose gradient centrifugation	Multidrug Resistance (MDR) cell line [22]
RAW 264.7	Paclitaxel	Incubation, electroporation, and sonication	MDR cell lines [23]
PFSK-1 cells, bEND.3, A-123 and U-87 MG	Rhodamine123, paclitaxel and doxorubicin	Incubation	U-87 MG cells and zebra fish embryo[24]

Main Text:

2. Isolation of Exosomes:

Exosomes are heterogeneous in terms of their size, composition, activity, and source, which makes isolation complicated. Low exosome purity is caused by the inability of most current isolation techniques to completely separate exosomes from multicellular vesicles produced by non-endosomal pathways and lipoproteins with comparable biophysical properties. Therefore, how to efficiently enrich exosomes is a major issue currently for different purposes and applications, several isolation techniques are chosen for various applications and purposes, with ultracentrifugation, size-based isolation techniques, polymer precipitation, and immunoaffinity capture techniques among the most often used [11].

2.1 Ultracentrifugation Technique:

Ultracentrifugation [UC] is currently the most widely used isolation technique, which is also referred to as the gold standard for exosome extraction and separation. It suitable for separating components of large-dose samples with significant differences in sedimentation coefficient [12]. The ultracentrifugation process is primarily divided into two steps: first, a series of continuous low-medium speed centrifugation to remove dead cells, cell debris, and large extracellular vesicles; second, to separate exosomes at a higher speed with a centrifugal force of 100,000 g; and finally, to wash the

exosomes with PBS to remove impurities like contaminating proteins. Studies have shown that the yield and purity of target exosomes are affected by centrifugation time, centrifugal force, rotor type, and factors [12, 14]. Density gradient centrifugation is used to increase the purity of exosomes. It is typically used in combination with ultracentrifugation to improve purity [15]

2.2 Polymer Precipitation:

Exosomes are often harvested by centrifugation under the conditions of polymer precipitation, which typically uses polyethylene glycol [PEG] as a medium. This is done by reducing the solubility of the exosomes. This method was originally used to isolate viruses. Initially, viruses were isolated using this technique. The polymer precipitation method is appropriate for analysing large dosages of materials and is very simple to use with short analysis times. False positives may occur, the purity and recovery rate is limited, and the created polymer is difficult to remove.

2.3 Size-Based Isolation Techniques:

This technique separates biological samples based on the size difference between exosomes and other constituents. The separation principle of size-exclusion chromatography (SEC) states that macromolecules cannot enter the gel pores and eluted along the gaps between the porous gels with the mobile phase, whereas small molecules stay in the gel pores and are ultimately eluted by the mobile phase. The biological features of the isolated exosomes are not considerably changed with their full structure and uniform size; however, they may be contaminated with other particles of a comparable size, reducing their purity [16].

2.4 Immunomagnetic Isolation:

Exosomes are seen in the majority of body fluids that exhibit common exosomal markers like CD9, CD63, and CD81. Targeting these markers with magnetic beads allows for the possible collection of exosome subpopulations. For exosome isolation and subsequent analysis, magnetic beads are versatile instruments[45]. Exosomes can be specifically collected by antibody-labelled magnetic beads, and the magnetic field used in immunomagnetic isolation will separate the trapped exosome from other materials [46].

2.5 Filtration:

To separate cells and large EVs in biological samples, commercial membrane filters or polycarbonate can be used. Ultracentrifugation is frequently used in combination with filtration techniques to separate exosomes from proteins, where Membranes are used to sieve cells and large EVs. 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 [44].

2.5 Other Isolation Techniques:

There is still no kit that can isolation the ideal exosomes from the mixture of samples However, the production and quality of the exosomes are low, and the kit itself is pricey. , since, exosome separation and purification technology is continuously explored, developed, and innovated, more and more innovative approaches are becoming available for researchers to improve traditional technologies. A recent study produced a new micro-vortex chip that is integrated with *Morpho Menelaus* butterfly wings modified with lipid Nano probes to address the challenge of successfully isolating and purifying multicellular vesiclesfrom biological bodily fluid that passes through this chip. The generated micro vortices can increase the interaction force between the multicellular vesicles and butterfly wings while also inserting the probe into the multicellular vesicles to achieve high-throughput enrichment. Following testing, it was discovered that the separation efficiency of this technique exceeds 70% and has no effect on downstream analysis, indicating that it has potential application value. Although several methods for isolating and purifying exosomes have been developed, there are significant limitations that prevent them from meeting all needs. A combination of multiple isolation strategies may be more effective than a single method's separation effect.

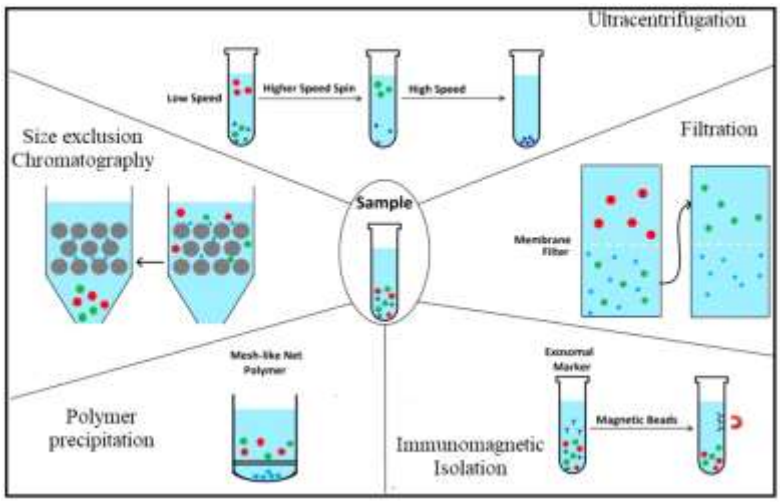

Fig 2 : Isolation technique of exosomes (ultracentrifugation, size exclusion chromatography, polymer precipitation, immunomagnetic isolation, filtration).

3. Loading of Exosomes on Drug:

The successful loading of exogenous drugs into exosomes is the key to exploiting exosomes as drug carriers; yet, this task poses a problem in evaluating the functionalization of exosomes as drug carriers. These medications are now loaded into exosomes by sonication, electroporation, transfection, incubation, extrusion, saponin-assisted loading, transgenes is, freeze-thaw cycles, thermal shock, pH gradient technique, and hypotonic dialysis. However, each method has its own set of pros and disadvantages.

Table: The Advantages and Disadvantages of drug loading strategies for exosome-based drug delivery systems

Methods	Advantages	Disadvantages
Sonication	Drugs loading and continuous drug release are both quite effective.	It causes exosomes to aggregate and modifies the structure of the surface protein.
Electroporation	The technique is easy to use and has been widely used to encapsulate siRNAs (Small interfering RNA) or miRNAs (MicroRNA).	This might cause RNA precipitation or exosome aggregation, reducing drug loading efficiency.
Transfection	It exceeds other approaches in terms of efficiency and molecule stability.	Transfection agents have toxicity and safety issues, and they may cause changes in gene expression in donor cells that produce exosomes.
Direct incubation	The packaging efficiency depends on the polarity of the drug	The encapsulation rate is relatively low.
Extrusion	This method has a high drug loading efficiency and produces exosomes of homogeneous size.	Researchers are unclear whether the mechanical force law will affect the properties of the released exosome membrane.
Saponin-assisted loading	It is highly efficient	Saponins are difficult to remove entirely and may cause an increase in exosome membrane permeability, cytotoxicity, and haemolysis on a continuous basis.
Freeze–thaw cycle	It is easy to use, employs modest conditions, and only rarely damages bioactive substances.	It can cause exosome aggregation and has a lower encapsulation rate than ultrasonic or extrusion.

Thermal shock	It has no effect on exosome shape and may increase exosome immunogenicity.	It affects the fluidity of exosome membranes as well as the cargo's stability.
pH gradient method	This approach is equally effective as ultrasonic or electroporation, and it has no effect on the stability of nucleic acid medications.	It lowers or aggregates EVs' overall protein content.
Hypotonic dialysis	The loading efficiency can be properly demonstrated.	It induces peak broadening and a shift in the size distribution

4. Application of Exosomes:

Recent retrospective studies on exosomes have mainly focused on the therapeutic aspects of cancer and inflammation, as well as on the molecular level of exosomes, mostly on cytokines and RNA. Exosomes can be secreted by both immune and non-immune cells [18]. Exosomes released by specific cells have the ability to act on receptor cells at distant sites, exhibiting many of the features of classical endocrine signalling [17].

4.1 Diagnostic Potential of Exosomes:

Exosomes can be present in all biological fluids and are released by all cells, making them desirable as less painful liquid biopsies with the possibility of longitudinal sampling to track disease progression. Exosome synthesis allows for the capturing of a diverse external and intracellular molecular cargo for multiparameter diagnostic testing. Exosome surface proteins also assist in immune capture and enrichment. Exosome diagnostic applications have focused on cardiovascular disease (CVD) [19-20], central nervous system (CNS) disorders [21], and cancer [23]. This strategy is rapidly expanding to include other disorders affecting the liver, kidneys, and lungs [24]. Some research has suggested that exosomes contain modest amounts of DNA(Deoxyribonucleic acid) and that this DNA can be useful in detecting cancer-related mutations in serum exosomes [25-26].

4.2 Therapeutic Potential of Exosomes:

Exosomes, either alone or as carriers for the delivery of their payload(s), are being investigated as therapeutic agents. Exosomes, unlike liposomes, are efficient in entering other cells and can deliver functional cargo with low immune clearance when administered externally in mice [27-31]. Furthermore, the therapeutic use of exosomes is promising because they have been shown to be well tolerated. Exosomes from mesenchymal and epithelial cells do not cause harm in mice when injected repeatedly [32, 33]. The use of (mesenchymal stem cells) MSC-derived exosomes in the treatment of a patient with graft versus host disease revealed that repeated injections were well tolerated and were not linked to significant adverse effects and resulted in patient response [34,35]. Other chemotherapeutic chemicals have also been loaded into exosomes, tried in animals for cancer therapy, with antitumor activity, and reduced toxicity reported. For example, paclitaxel-loaded macrophage exosomes increased lung tumour responses in mice [36].

4.3 Immunotherapeutic Role of Exosomes :

Exosomes are natural lipid bilayers rich in adhesion proteins that may easily interact with cell membranes and are well suited as drug transport carriers. As a result, the potential clinical applications of exosomes as biomarkers for illness detection and drug delivery carriers for therapy have received considerable interest in recent years [22].

5. Future Perspective:

Over the past few decades, despite major advances in understanding the secrets of exosomes, the challenges of efficient exosome isolation remain unsolved. This is due to the complexity of biological fluids, the significant overlap of physicochemical and biochemical features across exosomes, lipoproteins, viruses, and other extracellular vesicles, and the heterogeneity of exosomes themselves [37]. As a result, currently no single exosome separation technique is now accepted as appropriate for all investigations [39]. Even the gold standard ultracentrifugation method, depending on the biology samples used, is frequently contaminated with protein and lipoprotein impurities. Under these conditions, combining two or more approaches is a viable strategy for efficient exosome isolation, as evidenced by the previously reported combination of immunoaffinity-based exosome capture (or ultrafiltration) with density-gradient centrifugation [38, 40-41]. Researchers should carefully assess the strengths and drawbacks of the available methodologies for both diagnostic and therapeutic uses. Isolation of specific exosome markers (microvesicle markers) is also extremely beneficial in addressing the long-standing problem of quantitative exosome separation. On the other hand, the lack of an effective approach for isolating high-quality exosomes in bulk, limits the therapeutic applications of exosomes [42, 43].

6. Conclusion:

Exosomes' endogeneity is a natural and distinct advantage over liposomes, nanoparticles, microspheres, micro emulsions, and other synthetic drug loading methods. superiority of exosomes' makes them an essential channel for cell-to-cell communication, and they perform distinct bilogical functions in controlling regular living activities as well as in disease diagnostics and therapy. Exosomes have caught the attention of academics both at home and abroad as a current research hotspot. However, detection technologies for illness diagnosis and prognosis employing exosomal contents as markers have yet to be improved. The key aim is to increase the production and purity of exosomes, which has traditionally been the barrier restricting its transformation application. Recent studies have demonstrated that the proper combination of many methods for extracting and purifying exosomes can successfully solve the difficulties, and how to combine them to get the best results continues to be investigated further. Exosome loading capacity, as well as strategies for improving targeting, must be adjusted and improved. Conduct extensive and multi-disciplinary studies to study its biological processes and establish the foundation for future pharmacokinetics, toxicological, and clinical testing, which will assist to better understand the status of the body and diagnose and treat disorders.

List of Abbreviations :

BBB : Blood brain barrier.

MPS : mononuclear phagocyte system.

DC cells : Dendritic cell.

HEK : Human embryonic kidney cell.

HEK293 : Human embryonic kidney cell.

PEG : polyethylene glycol.

SEC : size-exclusion chromatography.

siRNAs : Small interfering RNA.

miRNAs: MicroRNA.

EV: Extracellular vesicles.

CVD : cardiovascular disease.

CNS : central nervous system disorders.

DNA: Deoxyribonucleic acid.

MSC : mesenchymal stem cells.

Acknowledgements

NA

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