International Journal of Health Technology
An Official Journal of **Kalam Institute of Health Technology**

REVIEW ARTICLE

Isolation, Purification, Characterization and Drug Loading Techniques of Exosomes: A Review

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Received: 21st February, 2022; Accepted: 03rd March, 2022; Available Online: 22nd March, 2022

ABSTRACT

Exosomes are extracellular vesicles having a bilayer membrane and works as a carrier. This is a newly emerging topic for targeted drug delivery. Exosomes have a natural ability to communicate with the other cells and exchange information, which proves to be an important characteristic in the aspect of drug delivery. The article embraces general introduction of exosomes and the comparison between various sources of exosomes such as plants, human origin, animal, microbial and potential sources with regards to their isolation and purification, characterization or analysis techniques and also includes the characteristics of obtained Exosomes.

Keywords: Centrifugation, Exosomes, Isolation and Purification, TEM, Western Blotting.

International Journal of Health Technology (2022)

How to cite this article: Bankar N, Kandisa RV, Chekuri DK, Sharma J. Isolation, Purification, Characterization and Drug Loading Techniques of Exosomes: A Review. International Journal of Health Technology. 2022;1(1):4-13.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Various methods such as liposomes, micelles, dendrimers, artificial nanomaterial carriers, etc. are established which can serve as carriers for the drug in targeted drug delivery. But these show shorter half-life in various other biological fluids, not efficient to cross the blood-brain barrier (BBB), the problem of immunogenicity, and as well as the risk of toxicity. Here exosomes gain importance, as they are naturally derived carriers by using exosome-related drug delivery so these are on-demand nowadays.[1]

CLASSIFICATION OF EXTRACELLULAR VESICLES

Apoptotic Bodies

Ranges from 50 to 5000 nm, which carry DNAs, RNAs, histone proteins, organelles, cytosol[2] and are produced by blebbing in the process of cell fragmentation[2] or apoptosis.[3]

Micro Vesicles (Microparticles/Ectosomes)

Ranges from 50 to 1000 nm, which carry proteins, lipids, nucleic acids, cytosol[2] and are generated by budding[2] and fission[3] of the plasma membrane.

Exosomes

Ranges from 30 to 100 nm, which carry proteins, lipids, nucleic acids, cytosol[3] and are generated by endocytosis, internal budding, exocytosis.[2]

CONTENTS OF EXOSOMES

Exosomes can be termed as small membrane-derived extracellular vesicles,[4] ranging approximately from 30 to 100 nm. Various cells secrete it as a mediator of intercellular communications, which carry genetic material , lipids and proteins as shown in (Figure 1). Its details are given in (Table 1). In various diseases, exosomes can play the role of biological markers.[5]

BIOGENESIS, RELEASE AND MECHANISM

Parent cell undergoes an endocytosis process that results in the formation of early endosomes (EE). These early endosomes

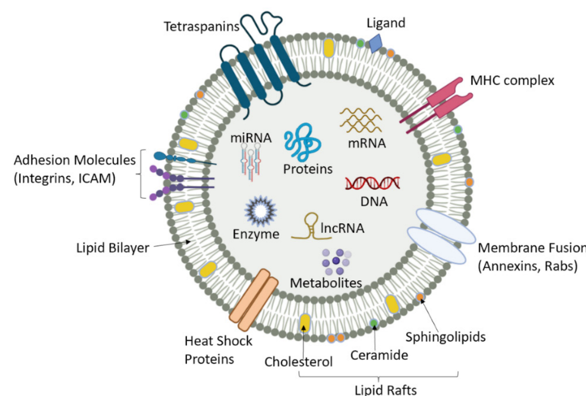

Figure 1: Exosomal Structure and Content

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further are transformed into late endosomes (LE). There takes place exchange of material between LE and Golgi bodies. In the cell, there are Multivesicular bodies (MVBs). And these MVBs contain many Intraluminal vesicles (ILVs). ILVs get bound to the membrane and get released terming as exosomes (Figure 2).[3,6]

The release of exosomes depends basically on the intracellular concentration of calcium as well as physiological, pathological conditions. Ligands attached to the surface of the exosomes get bind to the receptors present on the membrane of the targeted cell. By the process of budding and fusion, surface proteins along with the cytoplasm get transferred from the exosome to the target cell. Then after this genetic material and proteins are transferred horizontally.[3]

Exosomes can be isolated from various parts of plants such as fruits, flowers, seeds, leaves, rhizomes, etc. Tumors, various body fluids, cells belong to the human origin from which exosomes can be isolated. Even venom, milk, molluscs, ticks, bacteria, fungus, parasites, eggs, honey, etc can be used as a source of exosomes (Figure 3).

MANUFACTURING

Manufacturing of exosomes includes two steps upstream and downstream processing. The upstreaming process includes the cell source, culture condition media, cell expansion (culture substrate, bioreactor). Down streaming process includes filtration, the concentration of the conditioned culture media, isolation, and purification processes of exosomes.[3]

ISOLATION AND PURIFICATION TECHNIQUES

Isolation and purification techniques are inculcated to remove other unnecessary substances such as cell fragments or hindering substances. Some of those techniques are mentioned below:

- Centrifugation And Ultracentrifugation
- Membrane Filtration
- Vacuum Infiltration-Centrifugation
- Syringe Filtration
- Polymer-Based Precipitation/ PEG Precipitation
- Size Exclusion Chromatography
- Gel Filtration
- Isoelectric Precipitation

Centrifugation and Ultracentrifugation

- *Differential ultracentrifugation*

Series of differential centrifugal forces and times are used here. Firstly, larger particles are separated, then the smaller ones. Low centrifugal forces(≤10000 g) were used for the separation of cells, cellular debris, large EVs. Strong centrifugal force with a long time (100000-200000 g, ≥70 min) was used for exosome collection. Phosphate buffer saline is used to wash the pellet of exosomes, for the removal of remaining proteins. Purified exosomes, resuspended in PBS and stored at -80ºC.[7,8]

- *Density gradient ultracentrifugation*

Exosomes have a buoyant density that ranges from 1.10 to 1.20 gmL−1 in aqueous sucrose. So, they can be isolated by sucrose gradient centrifugation. It is based on particle density

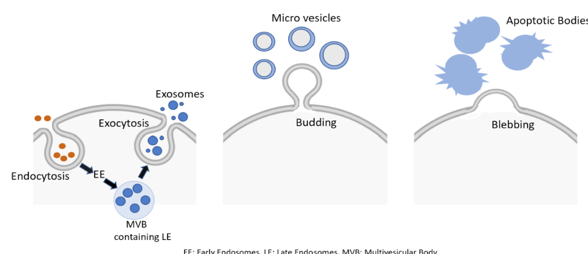

Figure 2: Release of extracellular vesicles

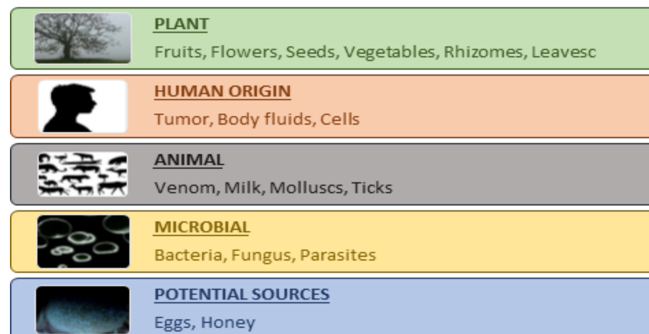

Figure 3: Different sources of exosomes

Table 1: Types of proteins, lipids, and nucleic acids are present in exosomes.[1,7,35,38,39]

Proteins	*Lipids and metabolites*	*Nucleic acids*
• Integrins,	• Ceramide,	• mtDNA
• Chaperones,	• Cholesterol,	• dsDNA
• Tetraspanins (CD9, CD63, CD81, CD82),	• Sphingomyelin,	• ssDNA
• Cytokinin,	• Phosphatidylserine,	• Viral DNA
• RAS related proteins	• Glycerophospholipids,	• mRNA,
• Heat shock proteins (HSC70, HSP60, HSP70, HSP80, HSP90),	• Glycerosphingolipids,	• miRNA,
• Transporters (ATP7A, ATP7B, MRP2, SLC1A4, SLC16A1, CLIC1),	• Phosphatidic Acid (PA),	• Pre-miRNA
• Receptors (CD46, CD55),	• Phosphatidylethanolamine (PE),	• lncRNA,
• Lipoxygenase,	• Phosphatidylcholine (PC),	• circRNA,
• ATPases,	• Organic acids,	• tRNA
• Aquaporins, etc.	• Alcohols,	• tsRNA
	• Steroids,	• snRNA, etc
	• Phenols,	
	• Amino acid conjugates,	
	• Sugar and conjugates, Etc.	

in sucrose density gradient solution. Gradual density decreases from bottom to top. Firstly, samples have to undergo the same steps as differential ultracentrifugation. Different EVs stay at different layers of gradient, in density gradient centrifugation. Better biological structure and properties of exosomes were obtained because of the cushioning effect of sucrose density gradient solution.[7,8]

Membrane Filtration

Different particles are isolated dependent on their molecular weight and size. Membrane filters are used for exosome separation. Normal prefiltration is carried out using a 0.22 μm membrane filter for removal of cells, cell debris, large EVs. Passing through the filter membrane exosomes are filtered and macromolecules are retained. 100 nm membrane filter is used in this process. Exosomes separated by this process have high purity.[8,9]

Vacuum Infiltration-Centrifugation

The sample is immersed in the specified buffer and then vacuum pulses are applied with intervals. The infiltrated sample is then recovered and dried, then subjected to further centrifugation.[10,11]

Syringe Filtration

The sample is filled in the syringe, which is then passed through two filters, called prefilter and filters of various pore sizes.[12]

Polymer-Based Precipitation/ PEG Precipitation

Here basically, the hydrophobic nature of exosomes contributes to precipitating it out, when we add a hydrophilic polymer. This is because polymer and water molecules get bounded. Process refers to the addition of Polyethylene Glycol (PEG) solution with the same volume to the different volumes of sample, from which exosomes are to be extracted. This mixture is further made up to a specific volume by adding Phosphate Buffer Saline (PBS), then subjected to further centrifugation processes.[9,13]

Size Exclusion Chromatography

It is filtration through a porous stationary phase which is based on the particle size. Porous stationary phase- spherical gel beads having many pores of a particular size. Thus, can be used to separate macromolecular particles. As the sample solution is passed through the stationary phase, small particles pass through pores and larger ones are eluted and are exited.[8]

Gel Filtration

The sample is subjected to filtration using the specific gel filtration columns, in which the void fraction of the column is collected and used further. This process is the same as Size Exclusion Chromatography, which is described above.[14]

Isoelectric Precipitation

Here the pH of the sample solution is adjusted according to the isoelectric point of the component that is to be aggregated and removed. Then the further process of centrifugation and filtration is carried out.[15]

CHARACTERIZATION TECHNIQUES

Characterization of the obtained exosomes is necessary to validate the isolation method. It is used to determine the purity, morphology, structure, size, sample concentration, particle size distribution, charge on particle, quantification of protein content, etc. Some of the techniques are mentioned below:

- Transmission electron microscopy (TEM)
- Immuno electron microscopy (ImmunoEM)
- Nanoparticle Tracking Analysis (NTA)
- Fourier transform infrared spectroscopy analysis (FTIR)
- Flow cytometry and fluorescence-activated cell sorting (FACS) analysis
- SDS page and western blot analysis
- Fluorescence microscopy
- Enzyme-linked immunosorbent assay (ELISA)
- Atomic force microscopy (AFM)
- Dynamic light scattering and zeta Analysis
- Scanning electron microscopy (SEM)
- Energy-dispersive x-Ray spectroscopy (EDX)

Transmission Electron Microscopy (TEM)

The size and morphology of the internal structure are studied in this technique. In this analysis purified sample is applied on formvar carbon-coated electron microscopy grids. Fixation of sample is done followed by washing, then Negative staining is done on grids. After drying samples are observed under an electron microscope. The chemicals, buffers, or solutions used for fixation, washing, or negative staining may differ according to the sample.[9,16]

Immuno Electron Microscopy (immunoEM)

As like TEM purified sample is applied on grids. Then blocking agent is used followed by washing. Here the grids are incubated with a specific primary antibody, then followed by an antibody conjugated to colloidal gold particles. Again, washing is carried out, grids are refixed and contrasted. Then after drying, observed under the transmission electron microscope. As with TEM chemicals, buffers or solutions used for blocking, washing, refixing or negative staining may differ according to the sample.[14,17]

Nanoparticle Tracking Analysis (NTA)

The Brownian motion of particles is tracked and images of particles at different views are obtained. For this analysis Nano Sight NS 3000 named instrument is used. Here we have to dilute the sample and observe under the microscope of this instrument, which also has a camera to determine the size distribution of sample particles.[9,18]

Fourier Transform Infrared Spectroscopy Analysis (FTIR)

In this analysis instrument named FTIR spectrophotometer is used. The sample absorbs radiation in a specific range and shows intensified absorption peaks at a particular wavelength.[19]

Flow Cytometry and Fluorescence Activated Cell Sorting (FACS) Analysis

Both these techniques resemble each other. The sample flows in a stream and one or more laser beams are used as a source, which detects the fluorescence and scattered light by the particles. FACS can sort the different types of cells by

distinguishing the cell characteristics. The sample is treated and reacted with specific reagents and then detected using a FACS Calibur flow cytometer.[20]

SDS Page and Western BLOT Analysis

The sample is run on Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane, where nonspecific binding sites are blocked. Then primary and secondary antibodies with HRP conjugation are incorporated, which are then detected in presence of substrate.[21,22]

Fluorescence Microscopy

Exosomes are labeled with fluorescent membrane dyes and cocultured followed by observation under fluorescence digital camera.[21]

Enzyme-Linked Immunosorbent Assay (ELISA)

We use an enzyme that converts the colourless substrate into coloured, which determines the absence or presence of antigen and antibody complex. The sample is treated and primary antibodies are added. Followed by washing secondary antibodies are added. Then after washing dye is added, which is then subjected to Optical Density measurement.[23]

Atomic Force Microscopy (AFM)

The surface scanning technique is involved in this analysis. The tip of the cantilever beam is used for the same. The relative size distribution is studied by this method. Sample pellets are taken and suspension is formulated by dilution in deionized water. Then is supposed to adsorb on mica sheets and is dried using a nitrogen stream. This is followed by scanning using Atomic Force Microscope.[9,17]

Dynamic Light Scattering and Zeta Analysis

The sample of purified exosomes is diluted and then filled into a cuvette by making up the volume with a specific buffer after which it is analysed using the DLS instrument.[24] The scattered light by the particles or exosomes is measured in this technique, which will give an idea about the size. For zeta analysis, the sample is subjected to a zeta potential analyser.[9,25]

Scanning Electron Microscopy (SEM)

The purified sample is dried using an incubator under specific conditions and then prepared with gold coating and observed under a scanning electron microscope.[25]

Energy-Dispersive X-Ray Spectroscopy (EDX)

EDX is generally combined with SEM, which produces good results. It studies the elemental pattern of exosomes.[25]

STORAGE AND STABILITY

Exosomes are stored effectively by cryopreservation, lyophilization, or freeze-drying. After usage, exosomes are cryopreserved by freezing, thawing, and often refreezing. The exosome activity is preserved by reducing the temperature below the threshold necessary for metabolic processes. Cryopreservation, on the other hand, maybe linked to exosome vesicle stress. This stress is caused by the production of intracellular ice and an osmotic imbalance during the freezing process. Although cryopreservation is advantageous in terms of ease and accessibility, temperature over -20⁰ C is not optimal for exosome preservation, and repetitive freezing and thawing may harm exosomes. As a result, exosome preservation by dehydration or lyophilization looks to be a potential option.

Lyophilization is a process of protecting exosomal formulations so that they may be maintained at room temperature for effective and long-term preservation. Because the elimination of freezable water associated with the exosomal content improves the stability of exosome contents (proteins or RNA), lyophilization is favored over freezing. Freezing (solidification) and primary drying (sublimation) are followed by secondary drying (desorption) in the lyophilization process. The core aspect concerned in the lyophilization preservation process is sublimation. Similar to cryopreservation, stresses associated with the freezing and dehydration phases of the lyophilization process may have a negative influence on the structural composition of exosomes.

Exosome vesicles and their payloads can be protected by including cryoprotectants or lyoprotectants in the formulation. Lyophilization can inhibit phospholipid hydrolysis, reducing the physical deterioration of vesicles over their shelf life. Furthermore, it may aid in the stability of the active components included in exosomes. Cryoprotectants are classified as Intracellular agents (dimethyl sulphoxide, glycerol, and ethylene glycol) and Extracellular compounds (sucrose, trehalose, and other sugars).[3]

DRUG LOADING TECHNIQUES

Exosomes are used to transport proteins, genetic substances and small molecules to the target cell. The lipid bilayer of exosomal membrane protects the cargo inside the exosome. However, the cargo is delivered to the target cell without its degradation. Some of those techniques are mentioned below:

- Sonication
- Electroporation
- Transfection
- Incubation
  - Direct
  - Indirect
- Extrusion
- Saponin assisted loading
- Transgenesis
- Freeze-thaw cycles
- Thermal shock
- pH gradient method
- Hypotonic dialysis

Sonication

The principle involves use of ultrasound to break the integrity of exosome membrane. During the ultrasound process the mechanical shear force comes into light, which is the main reason to break the membrane integrity of exosome by forming micropores. During the same process the drug is used along

with, which gets into the exosome through the deformed membrane. After this the exosomes are incubated which helps in regaining the membrane integrity. Sometimes when the drug is incorporated inside the vesicle and even on the outer surface of vesicle, sloe release and burst release of the drug is observed respectively.[3,6]

Electroporation

The principle involves use of electric field to create tiny holes on exosome membrane i.e., phospholipid bilayer. As holes are formed on membrane, its permeability gets increased. The drug is passed through it by diffusion mechanism and the exosome membrane regains its original structure on incubating.[3,6]

Transfection

The principle involves use of transfection agent to increase the permeability of exosome membrane. As the permeability gets increased the drug is entered into the exosome.[6]

Direct Incubation

The principle involves incubation of exosomes with the drug, wherein the concentration gradient plays the role for incorporating the drug into the exosomes. The drug is penetrated inside exosome through the membrane. Mainly the drug's hydrophobicity is considered as exosomes have lipid layer in their membrane. The drug and lipid interact with each other resulting in movement of drug inside vesicle.[3,6]

Indirect Incubation

The principle involves incubation of donor cells with drug, wherein the drug is incorporated into the cells. The drug is then moved into exosomes of that particular donor cells. These exosomes are then isolated, which already had the drug incorporated into them.[3,6]

Extrusion

The principle involves use of lipid extruder, wherein the mixture of exosomes and drugs is passed. The extruder has porous membrane of pore size 100-400 nm and has control on temperature. After the mixture of drug and exosomes is passed through extruder the membrane of the exosome gets disturbed and as result exosome gets loaded with drug by membrane recombination. Efficient drug loading is directly proportional to the number of extrusions carried out.[3,6]

Saponin-Assisted Loading

The principle involves use of saponins, that are membrane penetrating agents. These saponins form complexes with cholesterol present in the membrane. It dissolves the cholesterol and then create pores in the membrane, ultimately resulting into the increased membrane permeability.[3,6] After using or incubating saponin with exosomes it is necessary to purify the exosomes.[3]

Freeze-Thaw Cycle

The exosomes are incubated with drug at room temperature for particular time period followed by rapid freezing (-80º C/ liquid nitrogen), after this thawing process is carried out at room temperature. This involves membrane fusion mechanism. Minimum 3 cycles must be carried out for better drug loading.[3]

Others

In pH gradient method EVs are acidified internally to load negatively charged substance and pH gradient is formed in and out of exosomal membrane. Hypotonic dialysis method uses exosomes and cargo in PBS including a dialysis membrane. Thermal shock does not prove to be good method as it affects stability of drug and exosomal membrane.[6]

ROUTE OF ADMINISTRATION

Exosomes can be administered by different routes depending on the situation.[3]

- intravenously (common route for systemic administration),
- orally (convenient route of administration for patients),
- intratumorally (effective for antitumor therapy because EV retention time is more in tumors),
- intraperitoneally (used for administration of higher EV doses),
- intranasally (for delivering EV into brain as they have good BBB permeability)

ADVANTAGES AND DISADVANTAGES

Advantages Of Exosomes as Compared to Liposomes

- Exosomes have a greater ability to target than liposomes. Exosomes from many cell types include a variety of bioactive membrane proteins. Exosomes are a better candidate for targeted delivery of therapeutic compounds than liposomes because their specific surface proteins promote effective contact between exosomes and their target cells.[1]
- Exosomes are more stable in bodily fluid than liposomes. Liposomes can be recognized directly by macrophages and reticuloendothelial cells, or they can attach to serum proteins that interact with liposomes, causing liposomes' clearance. Exosomes, unlike liposomes, are much less prone to be phagocytosed by macrophages and microglia, indicating that they have better biostability.[1]
- Because of their endogenous origins, exosomes are extremely biocompatible vesicles. Exosomes are non-immunogenic vesicular carriers that escape the host's immunological activation or reduce the immunogenic response in the host. Liposomes, on the other hand, may elicit an innate immunological response following administration, leading to antibody formation against liposomal components or encapsulated cargos. As a result, exosomes may have superior biocompatibility than liposomes.[1]
- Exosomes inherit bioactive components from their parent cells that may be therapeutically useful. NPCs, for example, secrete exosomes with neurogenic potential that are enriched with pro-neural miRNAs, whereas activated microglia release exosomes that are specifically loaded with pro-inflammatory miRNAs and promote immunological responses. When compared to liposomes, superior

treatment benefits can be acquired by using therapeutically advantageous exosomes (e.g., MSC-derived exosomes) as drug carriers.[1]

Disadvantages of Exosomes as Compared to Liposomes

- Exosome yield is considerably lower than liposome yield. Exosome production is strongly dependent on the parent cells. The parent cells are the cells obtained from Human, animal, plant, microbial sources as mentioned in Figure 3. The diverse exosome secretion capabilities of cells, the high difficulty and cost of large-scale cell culture, and the time-consuming and inefficient techniques of exosome separation and purification all restrict exosome output, making industrial exosome production a non-ignorable challenge to overcome.[1]
- Exosomes have a low cargo loading efficiency. Exosomes are naturally filled with natural proteins and nucleic acids, which makes intended cargo loading much more challenging. Although there are methods to modify exosomes to increase loading capacity, the cargo loading efficiency of exosomes is still substantially lower than that of unpacked synthetic liposomes.[1]
- Exosome quality control is more difficult than liposome quality control. Quality Assessment parameters of exosomes involves Study of Morphology, Exosomal Content Detection, Exosomal Surface Protein Detection, Cytotoxicity Assays/Viability Assays, Microbial Enumeration Assays/Colony Formation Assay, etc.[1,26-28]
- Exosomes, even those produced by a single cell type, are exceedingly heterogeneous. We are unable to divide the heterogeneous exosome population into homogeneous ones due to a lack of sensitive high-throughput techniques for low copy number nucleic acids and proteins in the single-exosome dimension. Exosomes may also inherit undesirable macromolecules from their parent cells, as one of the exosomes' main jobs is to cleanse the cell of toxic or unwanted materials.[1,10]

APPLICATIONS

Therapy

Regenerative therapeutics: Intestinal wound healing, skin wound healing, ischemic pathologies (stroke and heart infarct), liver fibrosis, and sepsis can be the application of Exosome therapy.

Anticancer therapeutics: Tumor suppression, antioxidative action can be seen as effect of Exosome therapy.

Anti-inflammatory and hepatoprotective: Intestinal anti-inflammatory, hepatoprotection can be seen as effect of Exosome therapy.[29,30]

Biomarker

It is used as a biomarker to diagnose various types of diseases. Also, it can be used for the monitoring of the therapy. Cancer derived exosomes as body fluid-derived exosomes play an important role as a biomarker in early diagnosis as well as therapy monitoring. Urinary-derived exosomes are used to diagnose renal dysfunction and injury such as Polycystic kidney disease or diabetic nephropathy. Placenta-derived exosomes are used to diagnose pre-eclampsia. Serum-derived exosomes are used to diagnose glioblastoma, Parkinson's disease, Alzheimer's disease.[29]

Vaccines

Vaccines targeting pathogens with low immunogenicity e.g., N. meningitidis serogroup B (MenB) have been developed using Outer Membrane Vesicles (OMVs). This generates immunogenicity. The use of modified microbes improves the efficiency of vaccines.[29]

Exosomes as pathogen vaccines have a variety of potential benefits. These include: (i) more stable conformational conditions for the proteins; (ii) improved molecular distribution due to exosomes' ability to circulate in bodily fluids and reach distal organs; (iii) more efficient association with antigen-presenting cells due to exosomes' expression of adhesion molecules; and (iv) the fact that exosomes are one of the body's 'natural' mechanisms for transporting antigens between cells.[31]

Drug Delivery

Exosomes have the advantage of a nanocarrier. Exosomes generated from mouse lymphoma cell lines and loaded with curcumin have been proven to reduce inflammation in septic mice. Breast tumors are prevented by DC-derived exosomes carrying doxorubicin. Exosomes produced from cancer cell lines carry doxorubicin and paclitaxel across the blood-brain barrier. Exosomes generated from macrophages and monocytes were shown to be capable of transporting the antioxidant catalase to the brain in a mouse model of Parkinson's disease.[29]

Nutraceuticals

Exosomes, which have been shown to be taken up by mammalian cells and have beneficial effects on the digestive system, are made from whey and edible plants like grape or grapefruit. Furthermore, they are changeable in their cargo (for example, by adding doxorubicin or curcumin) that modifies their properties and gives a cost-effective and flexible alternative to exosomes generated from cell cultures.[29]

SUMMARY

Considering the Tables 2 to 6, various methods can be used for isolation and purification of exosomes from a single source. The size and yield of exosomes depend on the technique used and the type of source from which exosomes are obtained. Commonly used techniques for isolation and purification of exosomes are centrifugation, ultracentrifugation, membrane filtration. For characterization of exosomes, Transmission Electron Microscopy (TEM), Western Blotting, Nanoparticle Tracking Analysis (NTA) (Figure 4) can be used. For the characterization of plant-derived exosomes, TEM is the most commonly used technique (Table 2), but to date, there is no such standard technique developed for the isolation of exosomes from plants.[10] Heterogenous size exosomes are obtained despite using the same source. Finally, from the above study, the size

COMPARISON OF EXOSOMES' CHARACTERISTICS FROM VARIOUS SOURCES

Table 2: Exosomes obtained from plant source

Source	*Sample*	*Isolation and purification*	*Characterization/ Analysis*	*Characteristics of obtained exosomes*	*Ref*
Plant					
fruit	Strawberry	centrifugation, membrane filtration (0.45 µm pore size), ultracentrifugation	TEM	round or cup-shaped vesicles had yield 18 ± 3 µg and size (30–191 nm) from 250 mL juice	[32]
Flower	Tea flower	centrifugation, ultracentrifugation (Sucrose gradient of 8%,15%,30%,45%,60%)	TEM	spherical shape of size (20 nm)	[33]
Seed	sunflower seeds	vacuum infiltration-centrifugation, membrane filtration (0.5µm pore size), centrifugation	TEM	(50 - 200 nm)	[11]
Vegetable	cabbage	centrifugation, ultracentrifugation, PEG Precipitation, Size Exclusion Chromatography	NTA, TEM	spherical shape of size (100 nm)	[13]
Rhizome	ginger	membrane filtration(200µm pore size), centrifugation	different pH conditions, FTIR	pH ranges from 4 to 9 (294.1 d.nm, 343.8 d.nm,422.7 d.nm,518.2 d.nm,519.2 d.nm,545.9 d.nm) respectively	[19]
Leaves	*nicotiana benthamiana*	vacuum infiltration, centrifugation, syringe filtration(0.45 nm)	TEM	cup and round shape of size (30-220 nm) with the mean size of 117 ± 9 nm	[16]

Table 3: Exosomes obtained from a human source

Source	*Sample*	*Isolation and purification*	*Characterization/ Analysis*	*Characteristics of obtained exosomes*	*Ref*
Human origin					
Tumor	Breast cancer cell lines	centrifugation, membrane filtration (0.2 µm pore size), ultracentrifugation, ultracentrifugation (30% sucrose or D2O density cushion)	FACS analysis, western blot analysis, electron microscopy, fluorescence microscopy	the saucer-like shape of size (30–120 nm)	[21]
Body fluids	Plasma	centrifugation	NTA, western blot analysis	(115–200 nm)	[34]
	Saliva	centrifugation, ultracentrifugation	TEM, immunoelectron microscopy, ELISA, Western blot analysis, AFM	round shaped vesicles with bilayer membrane around it of size (30–120 nm) in clusters	[35, 17]
	Urine	centrifugation, ultracentrifugation, ultracentrifugation (30% sucrose/ D2O)	NTA, western blot analysis	(50–400 nm)	[36]
	Seminal fluid	centrifugation, syringe filtration (0.45 µm), PEG Precipitation, Ultracentrifugation	dynamic light scattering, zeta analysis, SEM, EDX analysis	(30-1000 nm) of size, yield by PEG was 9.166 mg/mL and UC was 7.564 mg/mL	[25]
	Amniotic fluid	centrifugation	SDS PAGE, western blotting, TEM	The inner diameter and microvesicle diameters were 123 nm and 180 nm respectively	[37]
	Breast milk	centrifugation	TEM, NTA, western blotting, ELISA	(150 nm)	[38]
Cells	Bone marrow mesenchymal stem cells	ultracentrifugation, centrifugation	TEM, Flow cytometry, western blotting	vacuolar cup having double membrane of size (50–150 nm)	[39]
	human placental Mesenchymal stem cells	PEG-based, ultracentrifugation	TEM, western blotting	spherical shaped with bilayer membrane of size (100–200 nm)	[40]

Table 4: Exosomes obtained from an animal source

Source	*Sample*	*Isolation and purification*	*Characterization/ Analysis*	*Characteristics of obtained exosomes*	*Ref*
			Animal		
Venom	*Gloydius blomhoffii blomhoffii*	centrifugation, gel filtration	western blot analysis, immunoelectron microscopy	(30–130 nm)	[14]
Milk	Raw bovine milk	centrifugation, ultracentrifugation, isoelectric precipitation	electron microscopy, NTA, Western blot analysis	(125–127 nm)	[15]
Molluscs	pacific oyster *Crassostres gigas*	centrifugation	TEM	(100–200 nm)	[41]
Ticks	*haemaphysalis longicornis*	centrifugation, membrane filtration (0.22 µm pore size), centrifugation (gradient concentrations 40, 20, 10,5 %)	electron microscopy, SDS PAGE analysis, western blot analysis,	spherical shape of size (100 nm)	[42]

Table 5: Exosomes obtained from a microbial source

Source	*Sample*	*Isolation and purification*	*Characterization/ Analysis*	*Characteristics of obtained exosomes*	*Ref*
			Microbial		
Bacterial	Lactobacillus	centrifugation, membrane filtration(0.45µm,vacuum), ultracentrifugation	AFM, NTA, dynamic light scattering and zeta potential, SDS PAGE	Closed double-membrane structures of size (10–300 nm)	[43]
Fungal	*candida albicans*	centrifugation, membrane filtration(0.45µm and 0.20 µm pore size),ultracentrifugation	TEM,NTA,SDS PAGE, western blotting	(100–200 nm)	[22]
Parasitic	*toxoplasma gondii*	centrifugation, membrane filtration(0.22 µm pore size)	electron microscopy, western blotting	intact continuous bilayer membrane of size (10–150 nm) with mean diameter 50 nm	[44]

Table 6: Exosomes obtained from potential source

Source	*Sample*	*Isolation and purification*	*Characterization/analysis*	*Characteristics of obtained exosomes*	*Ref*
			Potential sources		
Eggs	*Schistosoma japonicum*	centrifugation	TEM	(30–100 nm)	[45]
Honey	*apis mellifera* honey	centrifugation, ultracentrifugation	TEM, NTA, AFM	(<100nm)	[18]

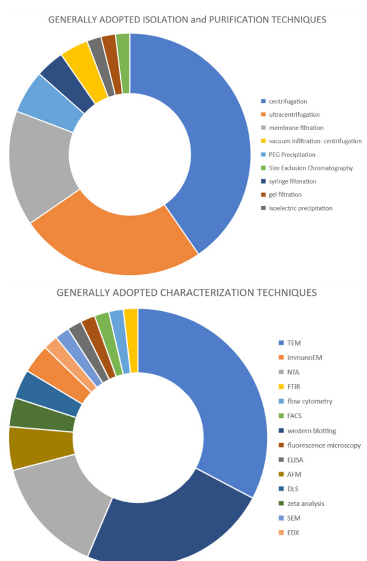

Figure 4: Generally adopted techniques of isolation, purification, and characterization of exosomes (as discussed above).

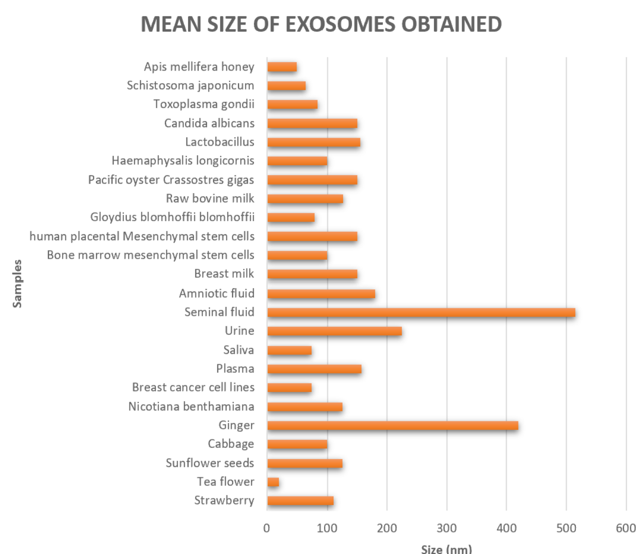

Figure 5: Graph representing the mean size of exosomes obtained

range of exosomes observed is from 20 to ~500 nm whereas 30 to 100 nm is most probable range. Human source-derived exosomes have a quite larger size than other sources (Figure 5).

CONCLUSION

Exosomes are obtained from various cell types (Figure 3), thereby have many opportunities for learning about their physiological processes and pathological conditions.[29] Exosomes are regarded as a promising delivery platform in biomedicine because of their low toxicity, low risk of immunological reaction, lengthy in vivo circulation, and nanosized dimension for deep tissue penetration, multiple cargo loading capabilities, as well as the capacity to alter surface molecules.[1] Without getting absorbed into body some nutrients pass the alimentary canal, here exosomes can function as nanocarriers to carry these substances to particular site/cell.[7] Exosomes have a wide range of applications covering a) therapies for regeneration and cancer, b) biomarkers for detection and therapy monitoring, c) vaccines, d) drug delivery to deliver antibiotic, proteins, anticancer drugs, e) nutraceuticals.[29]

Compared to mammalian source derived exosomes, non-mammalian source derived exosomes are not much studied. The traditional medicine would come into limelight if the exosomes obtained from natural products prove to be active and effective. Even though exosomes carry their own cargo inside, some of them are capable of carrying the therapeutic ingredients along with them. Such way they act as excellent nanocarrier. Exosomes from bacterial, plant, microbial source are termed as cost effective and can be scaled up easily as compared to the Mesenchymal Stem Cells (MSCs).[29]

Present hurdles in the development of scalable exosomes are suggestions for optimal separation procedures and effective drug loading methodologies. Before exosomes may be employed on a large scale in clinical trials, storage must be addressed as storage stability over time is a critical issue. Ensure that exosome-based medicines comply with regulatory standards for clinical approval. Regulatory implications for exosome-based treatments have not been adequately addressed compared to other forms of nanomedicines. Although research and clinical studies into the use of exosome-based therapeutics for drug delivery are still in their early stages, on-demand methods for advanced exosome understanding and systemic characterization will address the challenges and clinical transition issues of exosome-based therapeutics.[3]

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