Harnessing exosomes as a drug delivery system for neurodegenerative diseases

By

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A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.)

Department of Pharmacy
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August 2021

Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

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Approval

The project titled "Harnessing Exosomes as A Drug Delivery System for Neurodegenerative Diseases" submitted by Farhana Ferdous (ID 16346043) of Summer, 2016 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Hons.).

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Ethics Statement

The study does not involve any kind of animal trial and human trial.

Abstract

Exosomes are recently discovered bilayer containing membrane which are small-sized (30-100mm) nanovesicles that are formed due to the fusion of multivesicular bodies with the plasma membrane. Exosomes are involved in a number of functions including cell to cell signaling, unwanted proteins removal and pathogen transfer between cells. Exosomes are involved in promoting pathways of neurodegenerative diseases such as in Alzheimer's disease (beta-amyloid peptide), in Parkinson's disease (alpha-synuclein) and so on. All the exosomes take part in such enervative neuropathology, one encouraging aspect of exosomes is the development of a methodology to use these as natural delivery vehicles for therapeutics. Established characteristics including the blood-brain barrier crossing ability, half-life span, and stability compel it as an effective drug delivery system. The main focus of this review is the impact of exosomes in neurodegenerative diseases and disclose their probable role as a therapeutic tool for delivering drugs.

Keywords: Exosomes; Nano-vesicles; Transfer of pathogens; Neurodegenerative Diseases; Natural delivery vehicles;

Dedication

I want to dedicate this work to my parents who continuously helped me throughout my project.

Acknowledgement

This research could not also have been completed without the support of many people who are gratefully acknowledged here.

First and foremost, I would like to express my deepest gratitude and appreciation to my supervisor, Dr. Md Abul Kalam Azad (Assistant Professor, Department of Pharmacy, Brac University) for giving me this golden opportunity to do this wonderful project. His excellent guidance, priceless advice, insightful discussion, constant encouragement, profuse assistance, skillful suggestions, and continuous support throughout the thesis works allowed me to complete this research. With deepest gratitude, I acknowledge my guide for picking me up as one of his research students.

I take pride in acknowledging the intuitive guidance of Prof. Dr. Eva Rahman Kabir (Chairperson, Department of Pharmacy, Brac University) for sparing her valuable time whenever I approached her and showing me the way ahead. Her linguistic skill helped me to build up the capacity of expressing thought in a systematic manner.

My acknowledgment would be incomplete without thanking the biggest source of my strength, my family. I am thankful for the blessings of my parents.

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List of Acronyms

BBB	Blood Brain Barrier
EEs	Early Endosomes
LEs	Late Endosomes
MVBs	Multivesicular bodies
PEG	Polyethylene Glycol
ILVs	Intraluminal Vesicles
PDGER	Platelet Derived Growth Factor Receptor
hEGF	human Epidermal Growth Factor
SPIONs	Super Paramagnetic Iron Oxide Nanoparticles
TEMs	Tetraspanin-Enriched Microdomains
ICAM-1	Intracellular Addition Molecule
CTFs	c-Terminal fragments
APP	Amyloid Precursor Protein
CSF	Cerebrospinal fluid
SNPs	Single-Nucleotide Polymorphism

Chapter 1

Introduction

In recent years, the number of studies focusing on bioactive vesicles has remarkably increased especially work on exosomes. Exosomes are nanosized small membrane vesicles with a diameter of 30-150 nm. In multicellular organisms, communication between cells is maintained via extracellular molecules for instance nucleotides, lipids, or proteins. Besides these molecules, eukaryotic cells free part of their membrane as vesicles for communicating with cells (Schorey & Bhatnagar, 2008). Exosomes are secreted from diverse sites including dendritic cells, B lymphocytes, mast cells, T cells, and several epithelial cells, and found in various body fluids such as blood, urine, saliva, breast milk, amniotic fluid, and cerebrospinal fluid (Li et. al., 2018; Vella et. al., 2007). Depending on the origin, it contains a variety of substances such as non-coding RNAs, proteins and mRNAs which is known as "cargo" content and can deliver these contents to distal cells. Cellular functions are affected in either healthy or pathological ways by these cargoes. Surrounding or distal cells selectively takes up exosome and it can reorganize the recipient cells due to the active components of the cargo (Kalani et. al., 2014). Exosomes are formed by the fusion of an organelle of the endocytic pathway, the multicellular bodies (MVBs) with the plasma membrane, and the release of intraluminal vesicles (ILVs) as exosomes. The augmented interest concerning exosomes is due to their ability to contribute to pathogenesis and diagnosis (Hessvik & Llorente, 2018). Additionally, exosomes can also be used in therapy, where they can be used to deliver drugs or genetic elements (such as siRNA and miRNA) to target tissues, for example in neuroinflammatory diseases and gene therapy (Kalani et. al., 2014). Apart from this, exosomes that are derived from cells have the advantage of lower immunogenicity, inability to proliferate, and simple just preservation and transfer (Perets et. al., 2019). The aim of this review is to show the role and

function of exosomes in neurodegenerative diseases and their utilization as a drug delivery system.

1.1 Overview of Exosomes

Exosomes are a lipid-bilayer containing extracellular vesicles. Researchers are exploring new aspects of exosomes to modify it as a drug delivery system. A brief introduction of exosomes is given below-

1.1.1 Discovery of Exosomes

Extracellular vesicles were first discovered almost five decades ago. In 1983, within a week two papers were published, one by Harding, Heuser, and Stahl in JCB and another one by Pan and Johnstone in Cell which stated that in reticulocytes transferring receptors associated with small ~50nM vesicles are excluded from maturing blood reticulocytes into extracellular space. Under the electronic microscope, it was seen as a lipid bilayer with a spherical shape with a cup-like structure. Johnstone and his group in 1987 termed it as “exosomes". In 1997, it was found that B lymphocytes produced exosomes exhibit a number of functions, including antigen presentation, activation of T lymphocyte and immature cell function monitoring (Kang et. al., 2019). Gradually other functions of exosomes began to attract researchers. Exosomes became a valuable research sector after they found out about their content and ability to transfer them to cells. Functions of these vesicles comprises of cell-cell communication, unwanted proteins removal, and transfer of pathogens (Jan et. al., 2017). Nowadays, exosomes are playing a vital role in diagnosis, pathogenesis, and treatment purposes.

1.1.2 Biogenesis and Content of Exosomes

Complex processes are involved in exosome formation. Understanding the generation of exosomes and their content may help better for recognizing how cells utilize exosomes to communicate between cells and environment modification. The biogenesis process begins with inward invagination of clathrin-coated microdomains on the plasma membrane which produces early endosomes (EEs). EEs then come in contact with the Golgi complex to form late endosomes (LEs) or multicellular bodies (MVBs). Subsequently secondary invagination of multivesicular bodies occurs which leads to formation of intraluminal vesicles (ILVs) (Deng et. al., 2018). From here, the MVBs are degraded by fusion with the lysosomal membrane and releases ILVs into the lysosomes. Alternatively, the MVBs may fuse with the plasma membrane (Figure 1) releasing into the extracellular environment as exosomes (Bellingham et. al., 2012; Deng et. al., 2018). A number of studies revealed that the release of exosomes relies on RAB proteins including RAB11, RAB27, and RAB35. These proteins help in the trafficking of MVBs to the plasma membrane and in the secretion of exosomes (Deng et. al., 2018).

Exosomes are likely a heterogeneous population of vesicles. The content of exosomes differs based on the physiological state of the cell which produces exosomes and the mechanisms of sorting that control the packaging of vesicles. The content of exosomes contains various proteins, lipids and nucleic acids (Sarko & McKinney, 2017).

As exosomes are originated from endosomes, they contain membrane transport and fusion proteins like GTPase, annexins, flotillin, tetraspanins (CD9, CD63, CD81, and CD82), heat shock proteins, proteins involved in MVBs biogenesis, lipid-related proteins, and phospholipases. Some proteins like ALIX (ALG2 interacting protein X), TSG101 (tumor susceptible gene 101), and flotillin-1 are often present in exosomes which are known as marker proteins (Vlassov et. al., 2012). Exosomes also contain proteins for other purposes like cell-to-cell communication. The number of these proteins is over 4,400. Exosomes are often enriched

with various lipids such as cholesterol, SM, glycosphingolipids, and phosphatidylserine. These lipids are not only important for the structure of exosomes but also are essential players in exosome formation and release to the extracellular environment (Janas et al., 2015; Jan et al., 2015). The nucleic acid content of exosomes includes miRNA, mRNA, and other non-coding RNAs. Some studies have observed that exosomes contain the same miRNA content as their parent cell such as cancer cells (Valadi et al., 2007). Detailed information about exosomes contents can be found at Exocarta (www.exocarta.org). Table 1 reflects the major contents of neuronal exosomal vesicles and their characteristics.

Table 1: Exosomes' contents and their roles (Adapted and modified from Kalami et al., 2014).

Exosomes/biomolecules	**Examples**	**Role**
Transportation membrane and fusion proteins	GTPase, fibronectin	Mediates in membrane transport and also help in fusion with membrane
Tetraspanins	CD37, CD81, CD82	Helps to Morphogenesis, fusion processes with membrane
Heat-**s**hock proteins	Hsc70, Hsp27, alpha- B	This is a built-in attribute of exosomes
Proteins related to biogenesis of MVBs	Alix, TSG101	Produces multivesicular body and also used as identification markers for exosomes
Proteins attached to lipids and phospholipases	Phospholipase A2, phospholipase D, phosphatidylserine, ceramide, cholesterol	Regulates exosome steadiness and effective delivery, sending signals to cells and fusion to plasma
Genetic materials	miRNA, coding mRNA, and other non-coding RNAs, double-stranded DNA	Regulating the process of expression of gene, and transfer of information

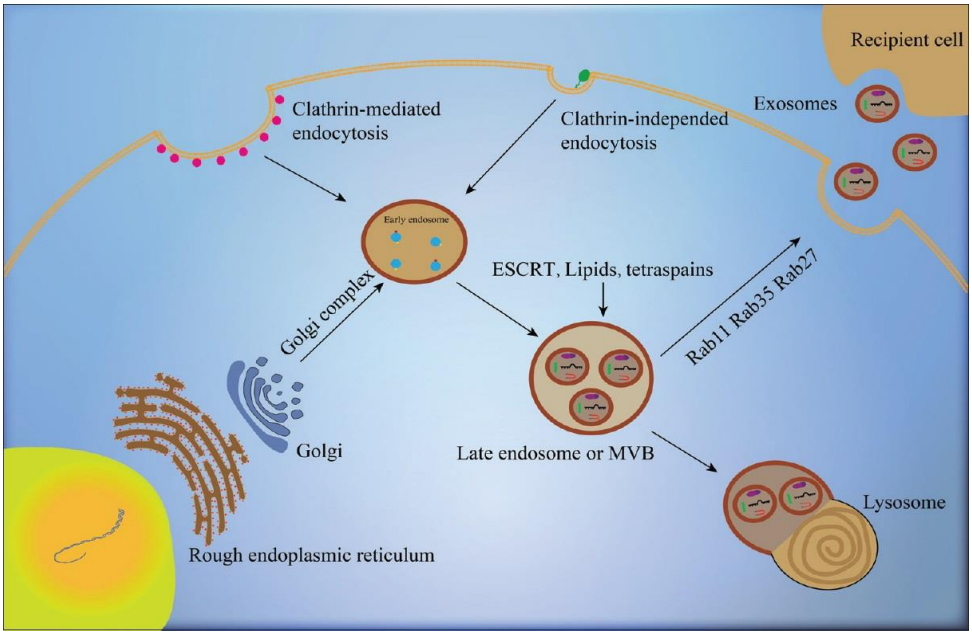

Figure 1: Exosome biogenesis and its release (Adapted from Li, Dong et al., 2018).

1.1.3 Exosomes in Neurodegenerative Disease

The process that governs the sorting of neurodegenerative disease-related proteins into ILVs could therefore pre-determine the fate of these proteins and thus play a role in disease progression. Nerve homeostasis, signal transduction, cellular communication is maintained by neuroglial cells which include different subsets and neurons (Mattson et al., 2003). In the CNS, different types of neuroglial cells such as microglia, astrocytes, oligodendrocytes, and neurons secret microvesicles into the extracellular environment which is also known as exosomes (Deng et. al., 2018). One of the vital roles of exosomes is in cellular communication. In the research of neurodegenerative diseases, this communication is a keystone due to interaction of surface proteins and target cell receptor and proteolysis of their cargoes and internalizations of their components via endocytosis (Jan et al., 2017). A common observation in neurodegenerative diseases is misfolded protein accumulation and forming inclusion bodies in specific brain regions (Li et al., 2012). This aggregation of proteins is an indication of neurodegenerative diseases (Figure 2). Obstruction in mitochondrial and proteasomal

functions, axonal transport and synaptic transmission, and enhanced endoplasmic reticulum stress is noticed in CNS due to their accumulation. The effect of the transfer of these infectious particles such as prions (in Creutzfeldt-Jakob disease, CJD), amyloid precursor protein (APP, in Alzheimer's disease, (AD)), alpha-synuclein (in Parkinson's disease (PD)) have brought light to previously in-identified pathways in neurodegenerative diseases. Table-2 shows some molecules found in exosomes related to diseases (Li et al., 2012).

Table 2: Contents of exosomes related to diseases (Adapted and modified from Bellingham et al., 2012).

Disease-associated Protein/ Peptide	**Normal Protein**	**Examples of Diseases**	**Association of These Proteins with Extracellular Vehicles**
PrP^{Sc}	PrP^{C}	Diseases include Creutzfeldt-Jakob, Fatal familial insomnia, Gerstmann-Straussler	Directly by transferring between cells
Aβ	APP	Alzheimer's disease	Directly by transferring between cells and also a small amount is observed in exosomes
α-synuclein	α- synuclein	Parkinson's disease	exosomes
Tau	Tau	Leads to Alzheimer's, frontotemporal lobe dementia and progressive supranuclear palsy	Not yet known
SOD1	SOD2	Leads to Amyotrophic lateral sclerosis	exosomes
PolyQ	Huntington	Huntington's disease	Not yet known

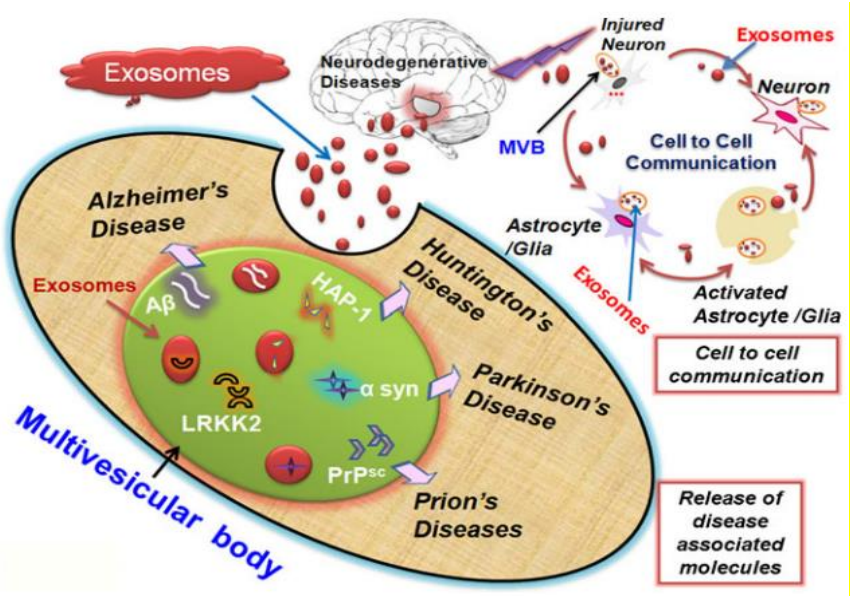

Figure 2: Multivesicular bodies release exosomes that contain disease-associated molecules. Neuronal toxins are also transferred to healthy cells via exosomes (Adapted from Kalami et al., 2014).

Exosomes are discovered only a few decades ago. Researchers are trying to use the potential of exosomes as a drug delivery vehicle. As exosome is a natural delivery vehicle, the chances of producing cytotoxicity are less. On the contrary, exosomes are also involved in transferring misfolded proteins. These proteins play a major role in producing a number of diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease, and so on. However, modified exosomes containing drugs produce a great impact on disease sites.

1.2 Objectives of The Study

i. To study the origin and biogenesis of exosomes.

ii. To analyze the role of exosomes in different neurodegenerative diseases.

iii. To explore the possibilities of using exosomes for diagnostic and therapeutic purposes.

Chapter 2

Methodology

Recent and relevant information is used in this review. The source of the articles from Google scholar, PubMed database, etc.Various terms related to these topics such as exosomes, neurological functions, neurodegenerative diseases, biogenesis of exosomes, methods of exosome isolation, preparation of exosomes for drug delivery, targeted delivery of exosomes, biological functions of exosomes led to a large number of articles. This review paper is a reflection of these articles.

Chapter 3

Preparation of Exosomes for Drug Delivery

This chapter reflects on how exosomes are prepared for the drug delivery system. Various isolation and purification methods, cargo loading methods are described here. Additionally, these cargos-loaded exosomes need to be targeted to the diseased site to produce the desired effect. A number of loading methods are also explained here.

3.1 Isolation & Purification

Nowadays, many biotech companies are considering exosomes as a delivery morality. The first step of using exosomes as a carrier is their isolation. Thus, for maintaining the physical, chemical, and biological functions of exosomes appropriate separation technique should be chosen. Various methods are utilized for the separation of exosomes controlled by their size, surface marker, and other characteristics. These methods include centrifugation, chromatography, filtration, polymer-based precipitation, and immunological separation (Table-3).

3.1.1 Ultracentrifugation

This technique utilizes different rates of sedimentation of components of various molecular weights. The centrifugal force is increased gradually. By using this method of isolated cells, vesicles, and proteins of different molecular weights can be separated and as a result exosome are separated. However, tiny molecules like exosomes are difficult to be separated by this technique. Therefore, for purification purposes, sucrose density gradient centrifugation is used along with ultracentrifugation (Konstantin, 2015).

3.1.2 Immunoaffinity Procedures

Immunoaffinity-based procedures such as immunoblotting are an important means for recognizing the separated exosomes. Exosomes which are separated by this technique are high in quality and purity. In this technique, coating of the magnetic beads is done with monoclonal antibody microparticles and specially bound to topical proteins of exosomes for desired isolation (Bunggulawa et al., 2018).

3.1.3 Size-exclusion Chromatography

This chromatographic technique is used to isolate molecules based on their size. Here, molecules are filtered through a gel. The small-sized molecules get trapped into the pores and their flow through the column is retarded according to their size. On the other hand, large molecules do not enter the pores and get filtered early. Chromatographic isolation is more advantageous than centrifugation as no shearing force is required in this technique (Nakai et al., 2016).

3.1.4 Ultrafiltration

Ultrafiltration is a semipermeable membrane best technique in which forces like pressure or concentration gradient leads to separation. This method can be used for the isolation of exosomes (Zhou et al., 2020). Most common filtration membranes have four sizes of 0.8, 0.45, or 0.22. exosomes that are collected using these membranes are larger than 800 nm, 400 nm, 200nm.

3.1.5 Polymer-based Precipitation

100 sums isolated using this technique usually is the size of 50-150 nm which is within the overlapping size ranges of exosomes and microvesicles. This process is done by mixing biological fluids with polymer-containing solutions (Zhou et al., 2020). This mixture is incubated at 4 degrees Celsius and centrifuged at a low speed. Polyethylene glycol (PEG) is the most commonly used polymer. This is a major drawback of this method (Hou et al., 2019).

3.2 Targeted Delivery of Exosomes

Most of the research has been done on the exosome delivery of therapeutic molecules. However, target-specific deliveries of therapeutic cargos are comparatively less studied. For delivering therapeutic molecules to target organs, surface engineering of targeted peptides or proteins on exosomes is necessary (Figure 3). The aim of bioengineering of exosomes is to achieve correct insertion and avoiding peptide cleavage (Jan et al., 2017). Exosome surface can be modified in many ways which are described below.

3.2.1 Transfection

Transfection is an effective method for loading cargos inter exosomes. Many studies have shown that transfection can be used to modify exosomes surface or membrane for desired delivery of exosomes contents. The method named calcium phosphate for commercially available lipofectamine which is a lipid transfection reagent used for transfection. As targeted delivery, exosomes were first tested on cancer cells. A good example of this targeted phenomenon is the successful delivery of let-ja to EGFR- expressing xenograft breast cancer tissue. In 2013, Ohno et al. modified donor cells which leads to a fusion between the

transmembrane domain of platelet-derived growth factor receptor (PDGER) and GE11 peptide. GE11 binding to EGFR on the exosomal surface was enhanced by PDGER-TD. This leads to the effective delivery of drugs to cancer cells (Ohno et al., 2013; Fu et al., 2020).

3.2.2 Assembly of Ligand on Exosomal Surface

Ligands are directly assembled on the surface of donor cells or exosomal surfaces to produce exosomes for targeting. The receptor bound to ligands helps in targeted delivery by forming a biological force. This method is comparatively beneficial because of its high specification, low cytotoxicity, and no impact on exosome size and shape (Fu et al., 2020). As exosomes are naturally designed cargo delivery system and biomimetically it is possible to put these in vivo, a number of targeted delivery system has been under study. Among them, the ligand overexpressed method is an effective one as it can enhance the drug delivery system. To justify this concept, researchers from Xiamen university engineered exosomes expressing human epidermal growth factor (hEGF) or anti-HER2 body on its surface. The aim was to target two types of tumor cells. They observed that biosynthetically displayed hEGF expressing exosomes gave higher biological activity and targeted delivery in comparison to another delivery system. Additionally, exosomes engineer to fuse with c(RGDyK) to form (cRGD-Exo) target to lesion region of the ischemic brain and deliver curcumin. Intravenous administration of this curcumin loaded exosomes repressed inflaming response and also produced apoptosis of cells. Thus, this method can be considered a promising way for targeted delivery (Liu & Su, 2019).

3.2.3 pH Gradient Derived Targeted Delivery

Studies have shown that protonation of exosomes to generate a pH gradient across exosomes surface can be utilized to increase the chances of targeted delivery (Antimisiaris et al., 2018).

This surface charge is a crucial property for internalization of components in cells, distribution in vivo, and aiming efficiently toward desire site. For example, in the case of tumor cells, they are more acidic due to their high intracellular glycolysis rate and production of lactate. Therefore, in this case, a pH-responsive drug delivery system can be used. Exosomes expressing intercalated motif (i-motif) which is a DNA stand rich in cytosine. It is used to target tumor cells and release DOX which is intercalated with a double-stranded i-motif/flare duplex. However, service charge less specific to exosome than ligand-receptor binding (Liu & Su, 2019).

3.2.4 Magnetism-guided Targeted Delivery

The method focuses on an exosome-based drug delivery vehicle that exhibits superparamagnetic behavior. It also produces a stronger response to an external magnetic field than individual superparamagnetic nanoparticles. For instance, in vivo study shows that drug-loaded exosome-based vehicle delivery enhanced cancer-targeting under an external magnetic field and suppressed tumor growth in hepatom 22 subcutaneous cancer cells (Shao et al., 2020). Jia et al. loaded superparamagnetic iron oxide nanoparticles (SPIONs) into exosomes which are fused with neuropilin-1 targeted peptide on its surface (Jia et al., 2018). These exosomes are able to cross the blood-brain barrier and target glioma specifically (Liu & Su, 2019). For effective delivery of chemotherapeutic drugs efficiently, magnetic attraction and ligand and receptor binding efficiency can be conferred to modified exosomes to enhance targeting ability. Different types of exosome based drug delivery system is shown in figure 3.

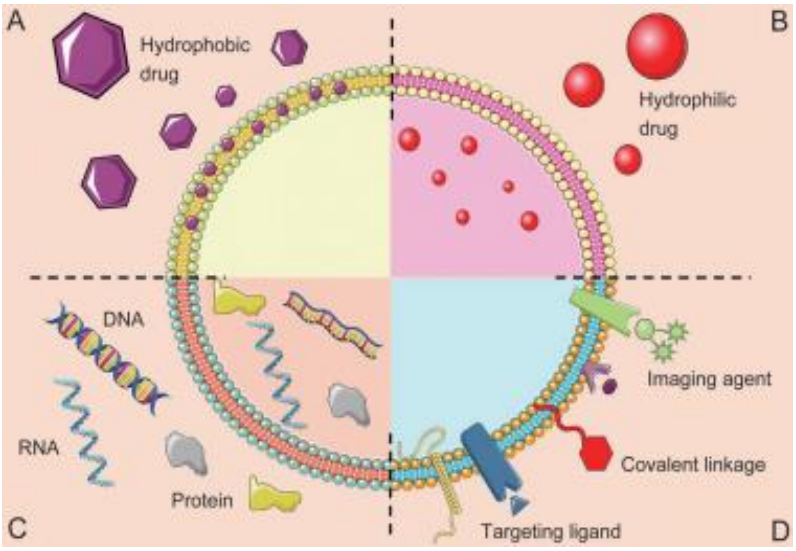

Figure 3: *The schematic representation of the different types of exosome drug delivery systems (Adapted from Luan et al., 2017).*

Table 3: Isolation methods of exosomes (Adapted and modified from Hung et al., 2013).

Methods of isolation	**Mechanism**	**Advantages**	**Disadvantages**
Differential centrifugation	The technique comprises of several centrifugation steps which remove cells, large vesicles, and debris and precipitate exosomes	It is a standard method and usually used to isolate exosomes from biological fluids and media	This method shows low efficiency when viscous biological fluids such as plasma and serum are used for analysis
Density gradient centrifugation	It is a combination of ultracentrifugation with a source or iodixanol, density gradient	Low-density exosomes are separated using this technique	Sensitive to centrifugation time
Size exclusion chromatography	This method consists of a column packed with porous polymeric beads.	Applicable for different solutions and exosomes separated using this	Running time is high

	Macromolecules are separated based on their size	method are not affected by shear force	
Filtration	This method is used to separate exosomes from proteins and other macromolecules by a membrane	Exosomes are separated from small molecules and soluble molecules	Less number of exosomes can be achieved due to adhesion to the membrane and applied force can damage it
Polymer-based precipitation	Biological fluids are mixed with polymer-containing preparations then the mixture is incubated and centrifuged	Mild effect on exosomes and use of neutral pH	Co-isolates non-vesicular molecules
Immunological separation	A number of immunological methods are applied in this method.	All types of exosomes can be isolated and they can also be used for the quantification of exosomes	Not applicable for large sample volumes and exosomes may lose their function
Isolation sieving	Exosomes are separated using a membrane then filtration is performed using pressure or electrophoresis	Less running time and gives highly purified exosomes	The recovery rate of isolated exosomes is low

3.3 Cargo Loading Method

In addition to exosomes' role in normal physiological processes and in disease, their therapeutic potential, and how they are released buy donor cells, it is also important to have knowledge of the process by which they are taken up by recipient cells. A key function of exosomes is the fusion with the plasma membrane and discharging its cargos to the cytoplasm

(Meldolesi, 2018). There are a number of ways how exosomes interact with plasma membrane which is discussed below.

3.3.1 Protein Interaction

Protein interaction is a physical contact established between two or more proteins as a result of biochemical events (Prada & Meldolesi, 2016). Tetraspanin which is abundant on the surface of the exosomes has numerous functions including cell adhesion, motility activation, and proliferation. CD9 and CD81 are tetraspanins which are well-known markers of exosomes which causes oocyte-spermatozoa and phagocyte fusion. In mini studies, it has been shown that the tetraspanins facilitate numerous viruses and parasites enter into the cell and replication. The plasma membrane contains receptor proteins which are transmembrane located in raft-like structures called tetraspanin-enriched microdomains (TEMs). These TEMs are actually tetraspanins which help in vesicular and cellular fusion. This leads to the theory that they have a role in exosome cell binding. Tspan8-CD49d complex on exosome surface was seen to be internalized readily by pancreatic and endothelial cells which might be due to interaction with the intracellular addition substance (ICAM-1) present on the surface of these cells (Andreu & Yáñez-Mó, 2014). Integrins are transmembrane receptors that transfer biological and mechanical signals across the plasma membrane and help in cell adhesion and migration. Another emerging role of integrins is related to the binding and internalization of exosomes. Integrins play an important role in transferring exosomal cargos to target cells. Exosome derived from different cell types carries integrin ligands including ICAM-1 or vascular cell adhesion molecule-1 which aids in binding to B2 and B1 integrins on the target cell.

3.3.2 Endocytosis

Endocytosis is defined as a process by which cells engulf substances from outside using a vesicle (Doherty et al., 2009). Most studies reported that endocytosis is used to internalize exosomes. After the initial introduction, it takes only a few minutes to get inside the cell (McMahon et al., 2011). Clathrin is a large protein with aids in the formation of a coated pit on the inner surface of the plasma membrane. These coated pits then bud into the cell and form a coated vesicle. CME requires assembled protein coat on the membrane to form curvature and also to form a spherical invagination (Koksonen et al., 2018). It is an active process it mediates in exosome internalization via the formation of clathrin-coated vesicles. These vesicles contain a number of transmembrane receptors and small ligands. For the release of contents, internalized vesicles are uncoated from clathrin proteins. Similar to CME, a small cave-like invagination known as caveolar vesicles are formed within the plasma membrane that is eventually reaches inside the cell (Sowa, 2012). There are a number of mechanisms other than clathrin-mediated endocytosis for exosome endocytosis in eukaryotic cells. These are rich in cholesterol, sphingolipids, and caveolins (Rennick et al., 2021).

Macropinocytosis is also known as cell-drinking uptake. In this process, the invagination membrane ruffles are formed and eventually they pinch off into the intracellular space. This process is similar to phagocytosis. It depends on rac1, actin, and cholesterol (Kerr & Teasdale, 2009). This process also involves Na+/H+ exchanger activity. Inhibition of Na+/H+ activity results in a significant reduction of oligodendrocytes-derived exosomes uptake by microglia (McKelvey, 2015).

3.3.3 Protein Interaction

Phagocytosis is a process which uses receptors that does not authorize direct interaction with the internalized molecules. Although primarily it is used to engulf bacteria and cell debris, it also appears as a potential method for exosomes uptake (Kwok et al., 2021).

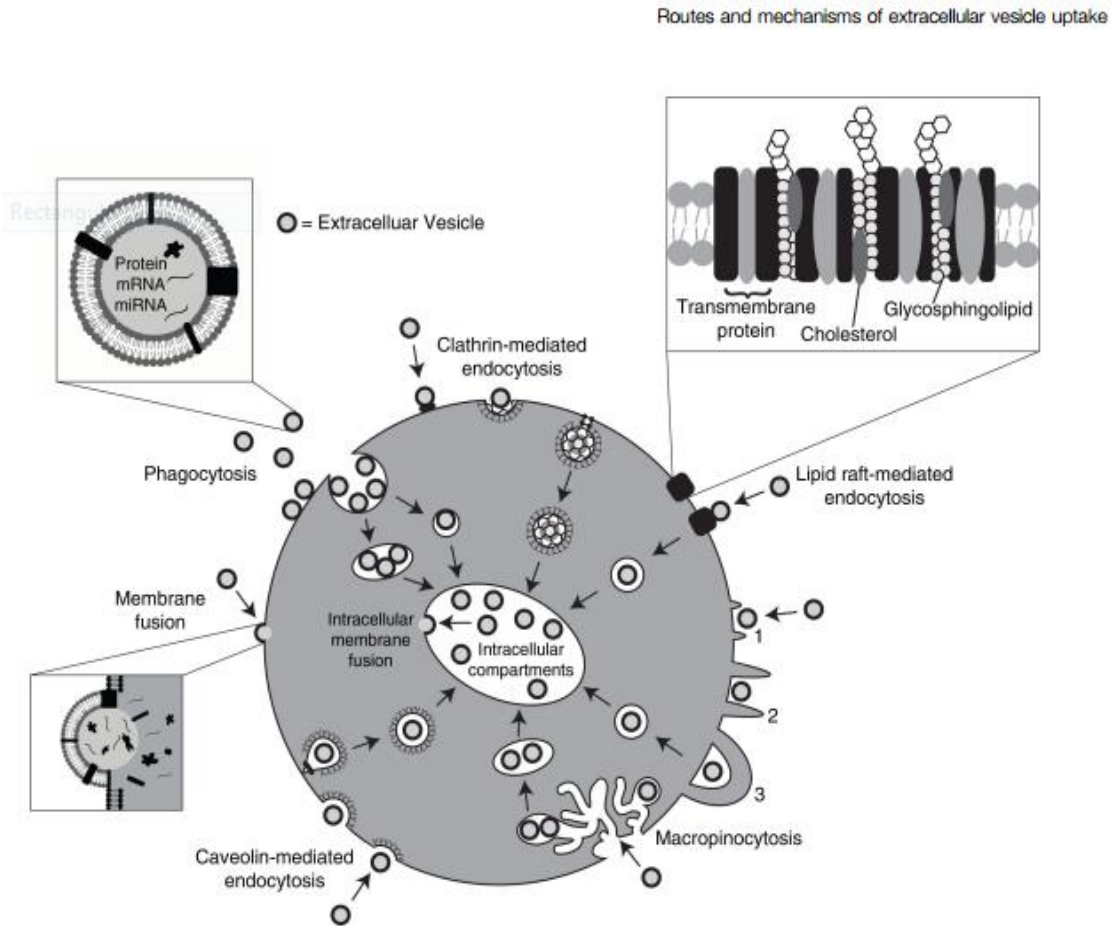

Figure 4: Membrane fusion methods of exosomes (Adapted from Mulcahy, V., 2014).

Various methods are involved in the isolation, purification, and targeted delivery of exosomes. Evidence suggests that exosomes produced by these methods lead to effective drug delivery as mentioned earlier. Improvements done recently in technical part of these methods have made the processes more effective.

Chapter 4

Effect of Exosomes in Different Neurodegenerative Diseases

Neurodegenerative diseases are defined as a heterogeneous group of disorders that produces symptoms including progressive deterioration of the central nervous system or peripheral nervous system (Przedborski et al., 2003). Alzheimer's disease and Parkinson's disease are the most common neurodegenerative disease. Exosomes play a vital role in these diseases. The pathological, diagnostic, and therapeutic roles of exosomes are briefly explained in this chapter.

As mentioned earlier exosomes are nanosized vesicles that occurs naturally. This vehicle comprises the abundance of adhesive proteins which leads to the rapid interaction with the cellular membrane (Kalani et al., 2014). Studies suggest that exosomes released from monocytes and macrophages can avoid entrapment in mononuclear phagocytes and at the same time enhances the delivery of incorporated drugs to target cells. Thus, increasing drug therapeutic efficacy (Haney et al., 2015). Exosomes derived from mesenchymal stem cells are also extensively tested for using them as a drug delivery vehicle. The results considered it to be safe in numerous clinical trials. Exosomes derived from mesenchymal stem cells are immunologically Inert. Moreover, exosomes are more suitable than liposomes. This is due to the fact that exosomes are less toxic and better tolerated in the body as evidenced by their ubiquitous presence in biological fluids. Exosomes also have the intrinsic homing ability. Additionally, these basic skills are amenable to in vivo and in vitro loading of drugs and their membrane can be modified to enhance tissue-specific homing (Lai et al., 2013).

Studies suggest that mesenchymal stem cells-derived exosomes are beneficial in a number of neurodegenerative diseases. For instance, 6- Hydroxydopamine (6-OHDA) containing exosomes are commonly used in Parkinson's disease because it triggers selective apoptosis of dopaminergic neurons (Gorabi et al., 2019).. Jarmalavičiūtė et al. reported that human dental

pulp-derived exosomes suppress apoptosis of dopaminergic neurons following treatment with 6-OHDA (Gorabi et al., 2019).

It was observed that exosomes containing curcumin and JS1124 were successfully delivered to the rodent brain via intranasal injection (Zhuang et al., 2012). These exosomes significantly reduced the inflammation mediated by LPS and experimental autoimmune encephalomyelitis induced by myelin oligodendrocyte glycoprotein (Kalani et al., 2014).

In another study, (Sun et al., 2010) observed that increasing administration of endosomal G226 by intranasal route suppresses glioma growth. These studies made the researchers more interested in using exosomes as a delivery vehicle, to treat neurodegenerative disorders and brain tumors (Jan et al., 2017).

4.1 Alzheimer’s Disease

One of the most common types of dementia is Alzheimer's disease and it is marked by two types of lesions: amyloid plaque and neurofibrillary tangles (Perl, 2010). Many studies have indicated that exosomes are involved in amyloid precursor and Tau proteins transfer and play a questionable role in Alzheimer's disease progression (Khachaturian, 1985). According to World Alzheimer's disease report over 50 million people are living with dementia and it will be shifted to approximately 150 million by 2050. Alzheimer’s disease is the leading cause of dementia in 60 to 80% of all cases (Lindsay et al., 2004).

4.1.1 Pathogenesis

Exosomes have extensive impacts on neuronal development activation and regeneration. This is due to the fact that exosomes are involved in intracellular transmission through the transfer

of their contents or encapsulate and abolishes cellular debris (Liu, 2019). Moreover, these vesicles have been spotted to spread misfolded proteins which lead to the onset and propagation of diseases. Studies suggest that neurodegenerative disease progression is done by transferring misfolded proteins to other healthy cells. This transfer changes the confirmation of properly folded proteins to misfolded conformation. There are several hypotheses about how these misfolded proteins are transferred. Among these, the idea that exosomes contribute to this lesion spreading is well established (Xiao et al., 2017). Patients with Alzheimer's disease amyloid beta-peptide is a major component of senile plaque. Aβ is produced due to the breakdown of amyloid precursor protein (APP) and its c-terminal fragments (CTFs). The hyperphosphorylated tau protein is a potential marker of Alzheimer's disease which is one of the important components of neurofibrillary tangles. Tau is linked to the discovery of a number of pathological conditions which are also known as Tauopathies. Two main isoforms of Tau in the adult brain are 3R and 4R. The imbalance of 3r / 4r is related to the decreased Tau functions which include tasks like transportation of components and maintaining stability of the structures of microtubules (Iranifar et al., 2019). There is a crucial need to discover new ways to identify and treatment due to the high incidence of Alzheimer's disease. Exosomes are observed to transfer toxic Aβ and hyperphosphorylated Tau between neuronal cells in cellular and animal models of Alzheimer's disease. They not only spread pathological proteins but also plays a detrimental role in impaired neuronal functions by various means in Alzheimer's disease. For example, exosome content induces apoptosis of neuronal cells in astrocytes revealed to amyloid protein and in 5XFAD mouse models of Alzheimer's disease (Yin et al., 2020). The classic hallmark of Alzheimer's disease is senile plaque. Aβ is released as a result of sequential cleavage of amyloid precursor protein (APP). Alzheimer's disease is an age-related neurodegenerative disease that is characterized by progressive memory loss and declining cognitive function. The knowledge on available treatment and existing reasons of

Alzheimer's disease is valuable in treating the disease (Cai et al., 2018). Studies have suggested that gene mutation is the precursor of toxic proteins. This includes amyloid precursor protein gene (APP) and presenilin which promotes Aβ production. In CSF of Alzheimer's disease patients, there is the presence of Aβ and Tau proteins suggests that toxic proteins may penetrate into the extracellular fluid through nerve cell secretion. Observations suggest that adding toxic proteins to the cell culture medium or CSF increases the intensity of pathogenesis and produces behavioral changes both in nerve cells and animal models analogous to Alzheimer's disease (Xiao et al., 2017)

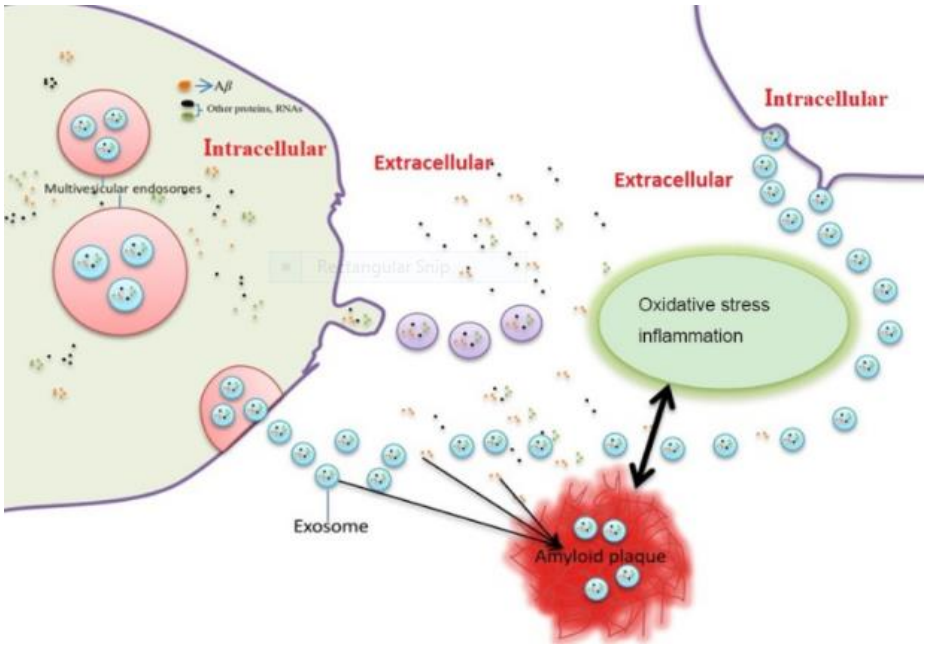

Figure 5: The emerging role of exosomes observed in beta-amyloid peptide pathology (Adapted from Xiao et al., 2017).

These results suggest that a major pathway for Alzheimer's disease progression after the initiation can be the transmission of toxic proteins between cells (Jiang et al., 2019). As exosomes transfer cargo between cells and the presence of toxic proteins related to the diseases is found in these vesicles, this might provide a new line of research for revealing the disease progressive pathogenesis and improving the current treatment status of Alzheimer's disease (Jiang et al., 2019).

4.1.2 Biomarkers

Biomarkers are necessary for a number of reasons including improving diagnostic sensitivity and specificity and also for the better understanding of biological activities of Alzheimer's disease. These biomarkers can help in the detection of Alzheimer's disease prior to apparent clinical symptoms manifestation. Lately, Alzheimer's disease is distinguished from other forms of dementia in the early stage by using the combination of CSFp-tau and CSF Aβ1-42. The concentration of p-Tau in exosomes is more helpful for diagnosis compared to CSF p-Tau concentration. This is due to the fact that p-Tau concentration in exosomes is more appropriate for identifying the degree and stage of Alzheimer's disease by combining symptoms based on a positive correlation between the amount of p-Tau in CSF exosomes and the severity of Alzheimer's disease. Studies has claimed that full length and mid-region Tau is present in high amounts in CSF and plasma exosomes of Alzheimer's disease patient then free solution where is full-length Tau is absent in CSF and plasma exosomes of healthy people. Several studies have suggested that detection of Aβ1-42 can bring forward the diagnosis of Alzheimer's disease by more than 15 years. But there are certain drawbacks of this method. For instance, in the beginning of Alzheimer's disease, the detection role is about 40 to 50%. Additionally, the plasma concentration of Aβ does not change over time in Alzheimer's disease patients. Thus, plasma Aβ cannot be utilized as an Alzheimer's disease biomarker. However, CSF exosomes containing Aβ are observed to improve the diagnostic sensitivity of Alzheimer's disease and the rate is 86% (Chen et al., 2017). The urge to utilize exosomes as a biomarker is due to the discovery of disease-related proteins in exosomes. Fiandaca et al. Observe that the level of p-Tau and Aβ found in exosomes can readily predict the development of the disease long before the onset. Liu and his co-workers observed in their experience that exosomal miR-193b could be a potential biomarker for detecting the early stage of Alzheimer's disease (Malm et al., 2016). miRNA released from exosomes modulates amyloid precursor proteins (APP) function

and their expression. Predicting biomarkers for the detection of the disease is critical so that preventative strategies could be applied to retard cognitive decline as pathological changes initiate years before the appearance of clinical symptoms. Distinct features of exosomal miRNA found in plasma and CSF of human subjects have prompted the potential application of miRNA as diagnostic biomarkers. It has been observed that expression of APP can be suppressed by overexpression of miR-193b. This suggests that the exosomal miR-193b has the potential to be a unique biomarker of the disease. Furthermore, a machine learning model predicted 7-miRNA as a signature marker for Alzheimer's disease with 83 to 89% accuracy (Chen et al., 2017). Unfortunately, the symptoms of Alzheimer's disease can be hidden for about 17 years (VanGiau et al., 2016).

4.1.3 Therapeutic Potential

Exosomes can act as a potent Aβ scavenger when administered intracerebrally. It binds to Aβ through enriched glycans on glycosphingolipids. These glycosphingolipids are present on the surface of exosomes suggests a role in Aβ clearance in the CNS. Exosomes provide novel therapeutic interventions for Alzheimer's disease by improving Aβ clearance (Cai et al., 2018). Microglia takes up the neuronal exosomes containing Aβ which contributes to peptide clearance from extracellular space and reduces Aβ pathology. Neuronal exosomes or exosomes isolated from hypoxic machine camel stromal cells when intracerebrally infused into APP transgenic mice were reported to decrease Aβ accumulation and amyloid deposition. The administration of exosomes declines the level of inflammatory cytokines. This reduction results in the improvement of memory and learning capacities in APP/PS1 transgenic mice (Cui et al., 2017). Enzymes like neprilysin and IDE are present in exosomes mediates in degradation consist of the beneficial role of these nanovesicles in Alzheimer's disease. As neprilysin-containing exosomes get internalized, ID leads to a reduction in extracellular and intracellular

Aβ levels. Treatment of BV-2 cells with statin increases the secretion of exosomes containing IDE. This increases Aβ clearance. There are other enzymes including Endothelin-converting enzymes and metalloproteinase also contributes to Aβ degradation. Exosomal protein cystatin c potentially exerts a tropical role in the brain and thus has a beneficial action against Alzheimer's disease. Studies have shown that in Alzheimer's disease patients there is an imbalance in cystatin c. Correction of this imbalance could be of therapeutic interest (Martins et al., 2021). Alvarez-Erviti et al. Observed that exosomes delivering BACE-1 siRNA to mouse model resulted in a 60% knockdown of the BACE-1 gene and also Aβ levels are reduced by 55% (Alvarez-Erviti, 2011). These exosomes specifically targeted B secretase which produces Aβ from its precursor protein APP. Furthermore, more exams have the potential to deliver drugs. As mentioned earlier, curcumin contained in exosomes can relieve the symptoms of Alzheimer's disease by inhibiting the phosphorylation of Tau proteins. It also helps to lowers the death neurons in both in vitro and in vivo models (Yin et al., 2020). In Alzheimer's disease, there is a significant reduction in the expression of the miR-24 family. Exosomes containing miR-29b when introduced in a rat model of Alzheimer's disease produce overexpression of miR-29b. As a consequence, the downregulation of BACE-1 (β-site amyloid precursor protein cleaving enzyme) was confirmed in the transfected cells (Lakshmi et al., 2021).

4.2 Parkinson's Disease

Parkinson's disease is the second most common neurodegenerative disease after Alzheimer’s disease and it affects 2% of the population aged over 65 years (Mhyre et al., 2012). It is a progressive nervous system disorder. This disease leads to shaking, stiffness, and difficulty with walking, balance, and coordination. Symptoms start gradually, sometimes starts with little

or no expression. With the progression of disease, people may have difficulty walking and talking.

4.2.1 Pathogenesis

Parkinson's disease is a movement illness that worsens over time. The major pathological observation of Parkinson's disease is the presence of Lewy bodies. The most important component of Lewy bodies is the misfolded and fibrillary aggregation of alpha-synuclein. Other factors that contribute to the development of Parkinson's disease include oxygen species, neuroinflammation, cytotoxicity, apoptosis, and the loss of tropic factors. Exosomes containing alpha-synuclein act as a potential crucial figure in the spread of alpha-synuclein in neurotoxic form and it’s spread throughout the brain (Wu et al. 2017). Brain information, microglia activation, and secretory neurotoxic activities including reactive oxygen species are associated with Parkinson's disease. Samples collected from Parkinson's disease brain have shown a reduction in redox enzymes, catalysts, and superoxide dismutase, and other antioxidants (Haney, 2015). It has been seen that Lewy bodies begin in the peripheral nervous system and progressively move to the brainstem and then to the cerebral cortex. A theory has been proposed from these observations that Parkinson's disease can start in the enteric nervous system or the olfactory bulbs, then migrate to other parts of the brain as the disease progresses. Exosomes have a key part in Parkinson's disease development. The toxic form of alpha-synuclein is oligomeric alpha-synuclein which is widely found in exosomes (Danzer et al., 2012). Researchers demonstrated that exosomes with alpha-synuclein are more readily absorbed by recipient cells. Stuendl et al. observed that CSF of Parkinson's disease patients containing exosomes could induce the formation of alpha-synuclein oligomers (Stuendl et al., 2016). These observations suggest that exosomal alpha-synuclein is intimately involved in Parkinson's disease progression and transfer of alpha-synuclein oligomers between cells.

MicroRNAs from exosomes are also involved in Parkinson's disease pathogenesis (Jiong et al., 2019) reported that exosomal microRNA-137 is involved in the production of oxidative stress in neurons in Parkinson's disease (Yu, 2020). It is characterized by selective degeneration of dopaminergic neurons in the substantia nigra pars compact (SNpc) and the presence of Lewy bodies in the surviving neurons. Different genes have been linked to cases of Parkinson's disease including alpha-synuclein, LRRK2, Parkin, DJ-1, and PINK1. Most of this evidence supports the critical involvement of alpha-synuclein in the pathogenesis of the disease. The biological function of alpha-synuclein is unclear. However, they play a role in synaptic vesicles biogenesis and modulation of synaptic transmission. Gene mutation and single-nucleotide polymorphism (SNPs) produce alpha-synuclein oligomers which are more prone to misfolding and accelerates the formation of aggregates. These alpha-synucleins are linked to autosomal dominant forms of Parkinson's disease and increase susceptibility to sporadic Parkinson's disease (Russo, 2012). Injured neurons release exosomes containing alpha-synuclein transmitted from neuron to neuron leads to alpha-synuclein spread. On the other hand, the activation of an inflammatory response is caused by transmission from neuron to neuron. EVs containing miRNA, which is an inflammation mediator, are thought to activate TLRs, triggering an inflammatory cascade, according to various research. However, the exact mechanism of the incorporation of alpha-synuclein into exosomes is currently unknown. Emmanouilidou et al. suggested that neuronal cells secret alpha-synuclein in a calcium-dependent manner and this alpha-synuclein are exported to exosomes which exacerbates Parkinson's disease by interfering with the survival of surrounding healthy neurons at physiological concentrations. Grey et al. reported that certain exosomal lipids, such as ganglioside GM1 and GM3, cause neuronal amyloid proteins like alpha-synuclein to aggregate. Thus, exosomes not only act as they operate as molecular shuttles, but they also actively prime some of its components due to their lipid content and acidic environment. (Porro, 2019).

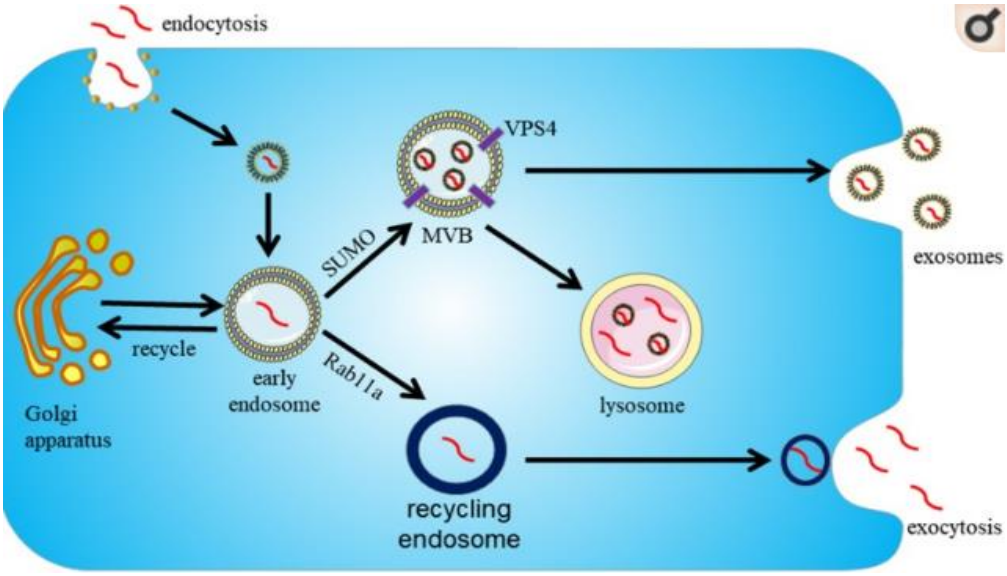

Figure 6: Schematic diagram of sorting α-syn into exosomes (Adapted from Yu, 2020).

4.2.2 Biomarkers

For the early diagnosis of Parkinson's disease, reliable diagnostic and prognostic biomarkers are urgently required (Niu et al., 2020). Biomarkers are useful indicators of pathological conditions for the effects of therapeutic interventions on disease progression. In the case of PD, early detection can be possible by tracking the biomarkers. Kitamura et al. In their study suggested that the expression level of three proteins clustering, complement C1n subcomponent and apolipoprotein (A1) may be considered as potential biomarkers for the diagnosis of Parkinson's disease. When compared to healthy persons, the expression of these proteins reduces in patients' brains. In addition, in patients, the fibrinogen gamma chain in plasma is reduced. Thus, these chains can also act as a biomarker according to the researchers (Kitamura et al., 2018). Gul et al. reported the presence of miRNAs in exosomes of patients with Parkinson's disease from the CSF. When compared to healthy controls, there were sixteen exosomal miRNAs upregulated and 11 miRNAs downregulated in samples. There was overexpression of miRNA-153, miRNA-409-3p, miRNA-10a-3p whereas in CSF, miRNA-1

and miRNA-19b-3p levels were considerably lower. Crucial pathways for Parkinson's disease such as neurotrophic signaling and dopaminergic synapses are affected by these miRNAs as they can target genes. These data suggest that exosomes containing miRNA have a potential diagnostic value in Parkinson's disease (table-5) (Porro, 2019). Yaoet al et. In their research found that exosomal miRNAs from cerebrospinal fluid act as biomarkers for Parkinson's disease. The included 52 patients in the study group and 48 healthy adults in the control group compared with controls Parkinson's disease patients have higher amounts of circulating exosomal miR-331-5p and miR-505. These molecules could potentially act as biomarkers for the disease (Yao et al., 2018). Niu et al. in their experiment observed that alpha-synuclein in plasma neuronal exosomes can be used as a diagnostic for Parkinson's disease diagnosis and as a predictive marker for Parkinson's disease development. They found that the amount of alpha-synuclein was significantly higher in patients with the early stage of the disease compared to healthy control groups (Niu et al., 2020).

Table 4: Diagnostic and therapeutic miRNAs in exosomes (Adapted and modified from Porro, 2019).

Therapeutic Potential	**Biomarkers**
miRNA- 155	miRNA-1
miRNA-7	miRNA-19b-3p
miRNA-124	miRNA-153
miRNA-205	miRNA-409-3p
miRNA-34b/c	miRNA-10a-5p
miRNA-7116-5p	miRNA-19b-3p

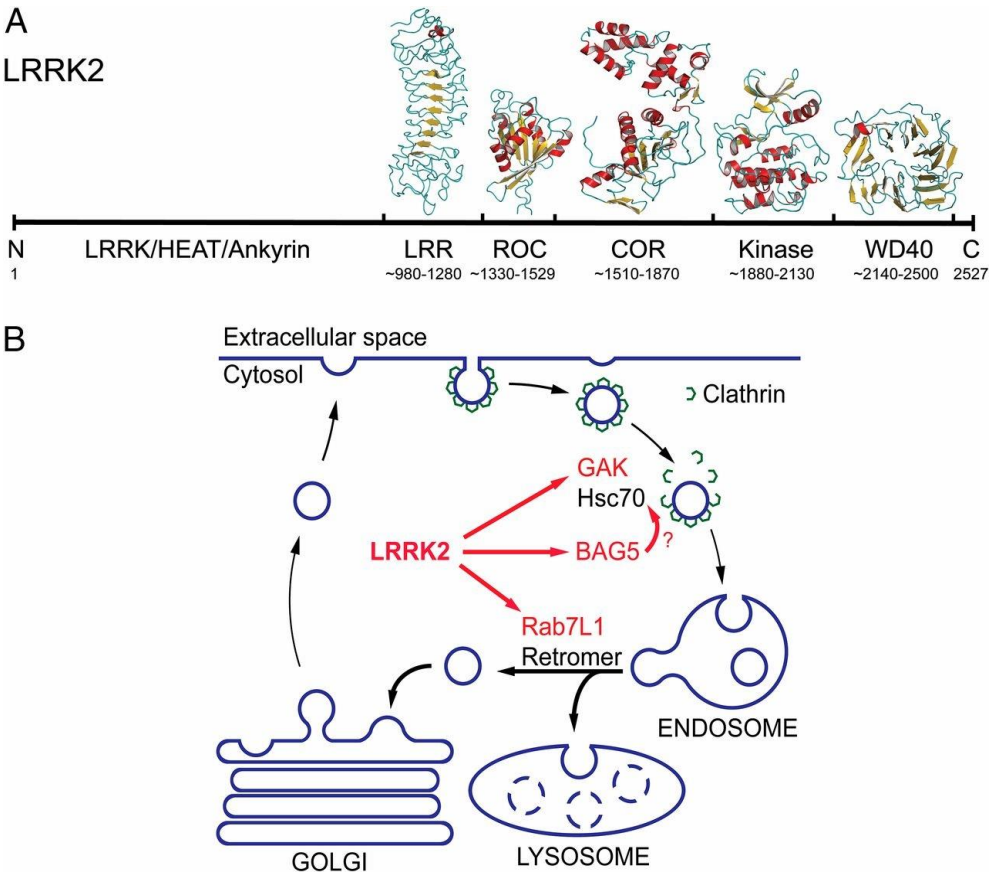

Figure 7: The relevant pathogenesis related to Parkinson's disease (Adapted from Luo, S., 2020).

4.2.3 Therapeutic Potential

In Parkinson's disease, the major application of exosomes includes drug delivery vehicles and diagnostic markers (Henry, 2015). Henry et al. reported in their study that in Parkinson's disease mice, brain-targeted catalyst-loaded exosomes that aggregated in neurons and microglial cells effectively reduced the release of inflammatory cytokines and significantly reduced neuronal mortality (Henry, 2015). Cooper et al. observed that exosomes containing siRNA were found to drastically lower alpha-synuclein mRNA expression levels. (Luo, 2020). In the case of 98% protein drugs which have the potential for many diseases fail in clinical tests because they are unable to pass the blood-brain barrier. Exosomes, which are naturally occurring nanosized vesicles that can pass the blood-brain barrier, have sparked interest as drug

delivery vehicles. (Table-6). When macrophages are transfected with plasmid DNA and mRNA, active catalase and nuclear factor κ-β, a transcription factor involved in encoded gene expression, were found in an experiment. Exosomes transfer these contents to neurons which results in de novo protein synthesis in target cells. The quantity of information available is lowered as a result of this transfer, and Parkinson's disease mice experience neuroprotection (Wu et al., 2017). Qu et al. examined the exosomes from the blood involving the transferrin-transferrin receptor interaction. They reported that exosomes loaded with dopamine produces more effective therapeutic efficiency in a Parkinson's disease mouse model and lowers systemic toxicity (Qu et al., 2013). Exosomes also represent a possible therapeutic tool in a diseased condition, beneficial to re-establish miRNA physiological levels like Parkinson's disease there are a number of miRNAs which is therapeutically related. For example, miRNA-155 is important for microglia's inflammatory response to alpha-synuclein in Parkinson's disease (Porro, 2019). Vilaca - Faria et al. Suggested that MSC-derived exosomes rescue dopaminergic neurons in the 6-OHDA mouse model of Parkinson's disease. In addition, alpha-synuclein aggregation can be inhibited, and NLRP3 inflammasome activation can be suppressed in the SNpc and striatum. As a result, the neuroinflammatory reaction in Parkinson's disease is ameliorated (Haney, 2015).

Table 4: Exosomes from different origins as biomarkers of diseases (Adapted and modified from Porro, 2019).

Origin of Exosomes	Test Subject	Outcome
CSF	Tested in PD patients and non-PD patients	upregulation of 16 exosomal miRNAs and 11 exosomal miRNAs downregulation occurs in Parkinson's disease patients
saliva	Tested in PD patients and healthy control	Elevated levels of phosphor α-Syn/total α-Syn is observed in the

		salivary exosomes is observed (P = 0.003).
CSF	Tested in 76 diseased patients and 58 controls	Decreased extent of exosomal α-Syn is observed in patients (P < 0.05)
serum	Tested in 109 PD patients and controls	Downregulation occurs by MiR-19b; upregulation occurs by miR-195 and miR- 24 in patients
CSF or blood	Tested in PD patients and controls	Downregulation occurs by MiR-34b/c in patients
plasma	Tested in PD patients at Hoehn and Yahr (HY) stages II and III and control group	In PD patients three proteins (apolipoprotein A1, complement C1r subcomponent, and clusterin) significantly decreased in patients (P < 0.05).
urine	Tested in PD patients and control group	LRRK2 levels increase in some PD patients
serum	Tested in patients in the early stage with essential tremor (ET) and controls (HCs)	Lower levels of α-Syn are observed in PD patients (P < 0.05)
serum	Tested in patients with dementia	miR-384 is upregulated
plasma	Tested in patients and controls	α-Syn is increases in patients (P < 0.0001).
CSF	Tested in 30 sporadic patients and 16 healthy persons	Decreased level of T-tau and p-tau is observed in PD patients.
plasma	Tested in 60 patients and 37 healthy persons	The level of neuron-derived exosome increases significantly in patients (P < 0.01).

All of this gathered information suggests that exosomes can be modified to target cells such as neuron or even specific neuronal parts and be successfully utilized for treating different types of neurodegenerative disorders.

Chapter 5

Advantages and Disadvantages

Exosomes possess a natural homing ability. Thus, it is less targeted by the immune system of the body. Exosomes also stand out among other vehicles for delivering both hydrophobic and hydrophilic drugs (Lee et al., 2020). These exams are more stable upon freezing and thawing compare with cells biocompatible and non-cytotoxic cells (Zaldivia et al., 2017). The autologous use of exosomes opened the door to an opportunity to produce personalized medicine (de la Torre et al., 2020).

Drugs given alone to the body possess certain disadvantages including inadequate water solubility, low biocompatibility, high rate of metabolism in the body, easy accretion in non-disease tissues, inability to penetrate the cell, and so on. For solving these problems drug delivery vehicles came into research. Exosomes are a suitable choice because of the characteristics. For instance, the immunogenicity of exosomes is extremely low compared to other carriers. The outcome of it is low cytotoxicity. Similarly, increased stability of exosomes in the blood is noticed. This is due to the fact that the surface of exosomes is derived from cells. They are not easily identified by the reticuloendothelial system. Therefore, the half-Life of exosomes in the blood is higher. One of the important reasons to use exosomes is the high efficiency of drug delivery. Exosomes exhibit high efficiency because of the effective protection of the loaded drugs. The natural content of exosomes also has certain curative effects on some lesions. For instance, it was experimentally confirmed that exosomes containing miR-150 derived from epithelial cells of tubules initiate the triggering and rapid growth of fibroblasts in the ischemia-reperfusion mice model. Exosomes can also be modified to contain signaling molecules when delivering drugs which leads to fusion with targeted recipient cells (Peng et al., 2020). Other advantages include their small size for penetration into deep tissues,

slightly negative zeta potential for long circulation, deformable cytoskeleton as well as similarities with the cell membrane (Luan et al., 2017).

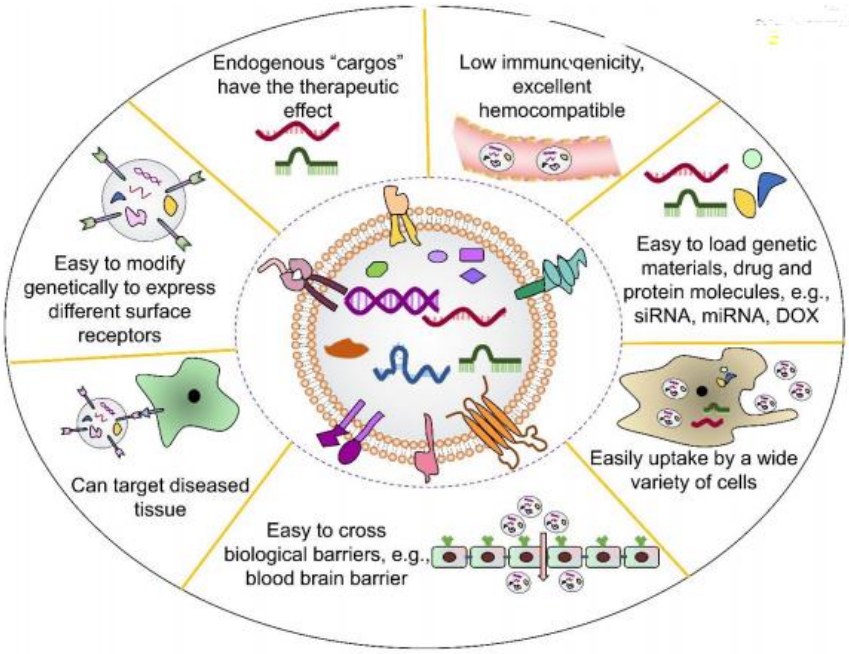

Figure 8: Advantages of exosomes as a therapeutic carrier (Adapted from Luan, X., et al., 2017).

There are certain barriers that need to be overcome for effective drug delivery. One of the major problems is nonspecific biodistribution in intended organs. These drugs containing exosomes are mainly accumulated in the liver spleen lungs kidney and pancreas. However, evidence suggests that unmodified exosomes derived from specific cell sources produce higher accumulation in certain tissues or organs than traditional drug delivery carriers. Moreover, rapid clearance from blood circulation is a major challenge for exosome-based drug delivery applications despite possessing a unique path and protein composition. This rapid clearance or cause due to macrophage capture. This challenge can be overcome by modifying exosomes. For instance, the introduction of polyethylene glycol (PEG) into exosomes leads to extended circulation time of exosomes (Wang et al., 2020). Other barriers include isolation and purification methods of exosomes, drug and antigen loading into exosomes, and targeting processes. A major challenge for bringing exosome technology into the clinic is the lack of

effective standardization isolation and purification method. It has been reported that combinations of isolation methods produce better results. However, these methods are complex and require intensive labor. Researchers are also trying to develop new strategies to load cargos into exosomes. Currently, the major types of loading methods are incubation, electroporation, and sonication. A recent study revealed a comparison of efficiency among the loading methods mentioned above. It was reported that the loading efficiency of sonication is 29% while 1.5% and 5.3% were h used for the incubation and electroporation methods respectively. Although there are certain barriers, exosomes are ideal systems for delivering therapeutic due to their characteristics (Yang & Wu, 2018).

Chapter 6

Future Direction and Concluding Remarks

For achieving remarkable success in this sector there are still many challenges ahead. Large-scale production of exosomes is one of them. To overcome this problem, rapid purification could be a solution. However, this technique needs further testing with different types of cells (Luan et al., 2017). In this context, exosomes derived from embryonic stem cells (ESC) and induced pluripotent stem (iPS) cells hold grape potential. Furthermore, for eliminating immunogenicity exosomes from stem cell-derived neurons or other brain cells can be used. These exosomes display intrinsic neurotropic behavior and enhance brain specificity. Another obstacle is producing exosomes that meet clinical-grade standards. This requires stringent and powerful characterization and purification methods to ensure a homogeneous population devoid of other biological entities (Lakhal & Wood, 2011). Exosomes regardless of their type (engineered exosomes or exosome mimetics) have to overcome these obstacles like the piling efficiency of drugs, the blood flowing time presuming that this determines their targeting efficiency, the intrinsic aiming efficiency of the targeted system, and how various engineering methodologies affect it. Artificial exosomes for exams-inspired liposomes can be developed to avoid potential safety problems which are related to allogenic vesicles from any source. These problems include the poor output and time requirement for producing vesicles from different sources. In reality, the research required for making this system had been done. For instance, optimization of microfluidic-based approaches has been done and GMP-compatible equipment for Kerala manufacturing is available. On the other hand, their high targeting efficiency like liposomes and their circulating time for reaching in vivo targets needs to be verified (Antimisiaris et al., 2018).

Nanotechnology heralded a new chapter in drug delivery. For delivery of these medicines' exosomes act as a promising vector. In the research based on molecular technology, the study

of exosomes has become popular nowadays. However, the specific role of exosomes produced by different cell type is still ambiguous and need additional investigation. In this context, many studies have aimed to reveal the role of these tiny vesicles that were extracted from specific cells including macrophages, rhabdomyosarcoma (RMS) cells, metastatic cancer cells, malignant mesothelioma (MM) cells, osteoclast pancreatic cancer cells, bronchial fibroblast, and mesenchymal stem cells. Exosomes act as an ideal and competitive nanocarrier for the targeted delivery of drugs even with or without being altered. All the naturally obtained exosomes have useful delivery properties, modified exhaust homes have an encouraging time ahead in drug delivery research (Bunggulawa et al., 2018). From the above discussion, it is visible that continued exosome studies and research will open new doors and will also optimize aimed curative outlook in the treatment of a broad-spectrum of disastrous diseases (Sarko & McKinney, 2017).

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