Chapter 1

Biological Function of Exosomes as Diagnostic Markers and Therapeutic Delivery Vehicles in Carcinogenesis and Infectious Diseases

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Additional information is available at the end of the chapter

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Abstract

Exosomes are nano-sized vesicles that are formed during inward budding of multivesicular bodies and the maturation of endosomes. They are secreted by almost all cell types under normal, pathological, and physiological conditions. They are found in mostly all biological fluids, such as breast milk, blood, urine, and semen. Exosomes are involved in cell-to-cell communication through the biological transfer of lipids, proteins, DNAs, RNAs, mRNAs, and miRNAs. Exosomes are enriched in tetraspanins, enzymes, heat shock proteins, and membrane trafficking proteins. There are numerous techniques that are used to isolate, purify, and characterize exosomes from biofluids. Isolation/purification techniques include ultracentrifugation, filtration, sucrose density gradient centrifugation, etc. Characterization techniques include flow cytometry, electron microscopy, NanoSight tracking analysis, Western blot, etc. These techniques are often used to help principal investigators understand the properties and biological functions of exosomes. However, some of these techniques can be very complicated and challenging, resulting in various drawbacks. Exosomes can be used as potential carriers for therapeutics. Thus, they can serve as biomarkers to diagnosis various diseases that are associated with cancer, genetics, viruses, bacteria, parasites, etc. Therefore, with advances in science and technology, many innovative techniques have been established to exploit the biological properties of exosomes.

Keywords: exosome, extracellular vesicles, biogenesis, therapeutics, cancer, infectious diseases, drug delivery

1. The discovery of exosomes

In the early 1980s, researchers Pan, Stahl, and Johnstone discovered a complex mode of extracellular vesicle (EV) secretion while studying the loss of transferrin during the maturation of reticulocytes in blood [1–4]. EVs were believed to bud directly from plasma membrane fragments that were isolated from cultured cells and human bodily fluids [1, 2, 5–7]. The research group showed that small vesicles were formed by inward budding inside an intracellular endosome which lead to the formation of multivesicular bodies (MVBs) [1, 8–10]. The MVBs produce intraluminal vesicles (ILVs) and fuse with the plasma membrane, releasing their contents into the extracellular environment [1, 8, 9]. The ILVs were termed "exosomes" in the late 1980s by Johnstone [2, 9]. Since their discovery approximately 40 years ago [3, 4, 8], exosomes have gained tremendous attention due to their involvement in intercellular communication [11]. EVs were originally believed to be waste products of the cell [8, 12, 13]. We currently recognize EVs as much more.

2. Exosome biogenesis and secretion

Exosomes are generated in the endosomal membrane when the ILVs of MVBs are formed during the maturation of early and late endosomes [1]. During maturation, MVBs are fated for lysosomal degradation or fused with the plasma membrane which leads to the secretion of ILVs as exosomes [1, 14]. The generation of the ILVs in MVBs contains the lateral segregation of cargo at the endosomal limiting membrane [15, 16]. In addition, it involves the formation of an inward budding vesicle and the release in the endosomal lumen of the membrane vesicle containing a small portion of cytosol [15, 16].

The Endosomal Sorting Complex Responsible for Transport (ESCRT) mediates exosome biogenesis [1, 17–19]. ESCRTs consist of approximately 20 proteins that are divided into the ESCRT-0, -I, -II, and –III complexes [20, 21]. These complexes contain ubiquitin-binding subunits [18, 21, 22]. The ESCRT-0 complex identifies and sequentially binds to ubiquitylated proteins in the endosomal membrane [23]. The ESCRT-I and -II complexes are responsible for membrane deformation into buds with sequential cargo [21]. The ESCRT-III complex drives vesicle scission [21, 24].

ESCRT-0 contains the hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) protein [22]. HRS identifies ubiquitylated cargo proteins and other constituents in a complex that consist of clathrin, the epidermal growth factor receptor pathway substrate 15 gene, and signal-transducing adaptor molecule [18, 25]. Most importantly, HRS recruit tumor susceptibility gene 101 of the ESCRT-I complex [26]. ESCRT-I is then involved in the recruitment of ESCRT-III, through ESCRT-II or the ESCRT-accessory protein ALG-2 interacting protein-X (Alix) [26]. Lastly, the separation and recycling of the ESCRT machinery interacts with the AAA-ATPase vacuolar protein sorting 4 [19, 27].

Exosomes are secreted by many cell types during normal, physiological, and pathological conditions [14, 28, 29]. They are secreted from cancer cells [28, 30], platelets [31], neurons

[32], epithelial cells [28, 33, 34], dendritic cells [28, 35], B and T cells [28, 36], astrocytes [28, 37], endothelial cells [28, 38], mast cells [31, 39], and mesenchymal stem cells [28, 40]. Also, exosomes have been identified in most bodily fluids, such as nasal secretion [28, 41], blood [42], serum [28, 43], ascites [44], amniotic fluid [44], urine [28, 45], breast milk [28, 46], and saliva [28, 43].

Depending on the cell type, exosomes are mainly secreted by the constitutive release pathway and/or inducible release pathway [28, 47–51]. In the constitutive secretion pathway, proteins are sorted into vesicles in the Golgi and transported to the cell surface where they fuse with the plasma membrane via exocytosis. In addition, Rab guanosine triphosphatases (GTPases) [52, 53], hetrotrimeric G-protein [52], protein kinase D [52, 54], glycosphingolipids, and flotillin [52] are involved in this pathway. Specifically, several Rab GTPases have been shown to act as key regulators of the exosome secretory pathway [49]. Rabs are a large group of small GTPases that regulate protein transport via endocytic and exocytic pathways in all cell types [52, 55]. In addition, Rabs are involved in membrane trafficking (i.e. vesicle budding, membrane fusion, and the transport of vesicles along actin and tubulin) [53]. Rab GTPases are composed of approximately 70 distinct proteins [56, 57]. Common Rab proteins include Rab11, Rab27, and Rab35 [58, 59]. These proteins are all involved in the transport of endolysosomal vesicles toward the plasma membrane [60].

Rab11 was the first Rab GTPase reported study that involved exosome secretion in human leukemic K562 cells by Savina et al. [61, 62]. Specifically, Rab11 is involved in the recycling from an endosomal compartment to the plasma membrane [63–65]. Both Rab27a and Rab 27b function in MVE docking at the plasma membrane in several cancer cell lines *in vivo* and *in vitro* [49, 55, 66]. Rab35 mediates MVB docking or tethering in oligodendroglia cells as reported by Hsu et al.[58, 61]. They revealed that the inhibition of Rab35 leads to intracellular accumulation of endosomal vesicles and impairs exosome secretion [58, 61]. In addition, Hsu et al. showed that Rab35 localizes to the surface of oligodendroglia in a GTP-dependent manner, where it regulates vesicular density [58, 61].

Inducible secretion is regulated by many cellular processes [67–69]. This pathway is regulated by stimuli, such as heat shock, hypoxia [52], DNA damage [52, 67, 70], increased intracellular calcium release [52, 67, 70], thrombin [52], extracellular ATP [52, 67], and lipopolysaccharide 39 stimulation [35, 52]. In 2012, King et al. demonstrated that the release of exosomes in breast cancer cells is promoted by hypoxia [71]. In addition, they demonstrated the hypoxic response could potentially be mediated by the hypoxia-inducible factor-1α—a group of transcription factors that are targeted for degradation under normal oxygen conditions by the action of specific O_2−, iron- and 2-oxoglutarate dependent prolyl hydroxylases [71, 72]. Another study was reported by Hooper and colleagues in 2012 [73]. In their study, they investigated the inducible release of exosomes cultured from rat microglia cells treated with recombinant carrier-free Wnt3a protein- a family of cysteine rich glycoproteins that play a role in tumorigenesis and act as morphogens during development [69, 73]. They observed that these Wnt3-induced cells increased exosome secretion through a glycogen synthase kinase 3-independent mechanism [73]. The process of exosome biogenesis and secretion is summarized in **Figure 1**.

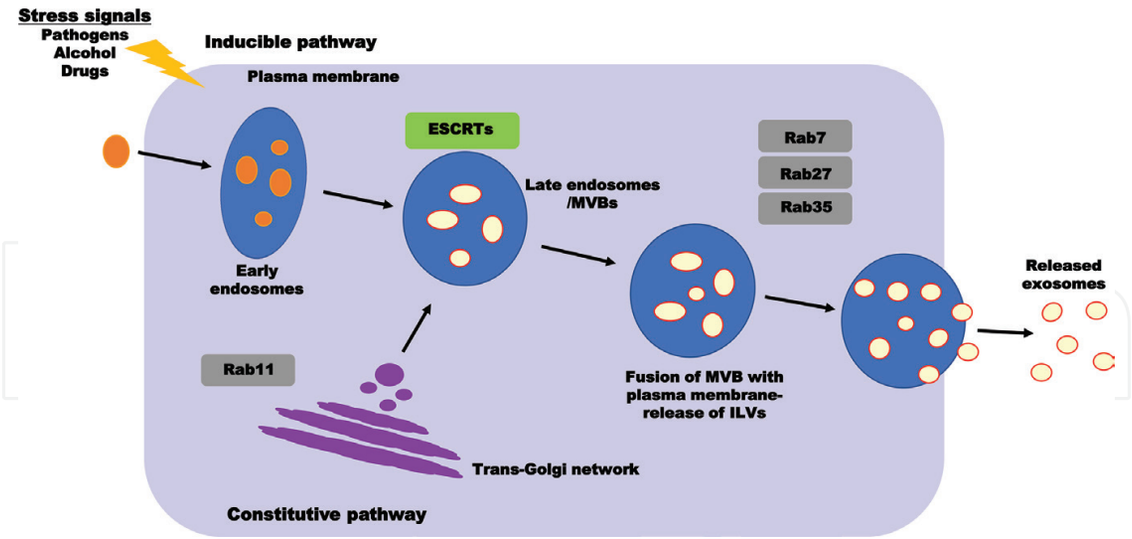

Figure 1. Biogenesis and secretion of exosomes. Exosome biogenesis and secretion is a complex process. Exosome secretion can occur by two different mechanisms, constitutive or inducible secretion. One or both of these pathways maybe operational depending on the condition of the cell. Constitutive exosome secretion occurs in various cell types under normal physiological and pathological conditions. Inducible exosome secretion is regulated by stressors (e.g. pathogens, alcohol, drugs). Multivesicular bodies (MBVs), intraluminal vesicles (ILVs), Trans-Golgi network and the Endosomal Sorting Complex Responsible for Transport (ESCRT) are four important compartments involved in exosome biogenesis and secretion. Rab guanosine triphosphatases (GTPases) (7, 11, 27, 35 etc.) are also depicted, they play an important role in exosome secretion.

3. Composition of exosomes

Exosomes carry a group of specific proteins, lipids, RNA, microRNA (miRNA), and DNAs, that represents their cells of origin [28, 56, 74], as depicted in **Figure 2**. Recent studies have shown that exosomes contain approximately 194 lipids, 4563 proteins, 1639 messenger RNAs (mRNAs), and 764 miRNAs [28, 75–77]. Exosomes are enriched in molecules, such as the major histocompatibility molecules (MHC) class I and II that play a key role in immunoregulation by processing antigenic peptides [28, 78]. Also, exosomes contain tetraspanins that serve as unique markers [79]. Tetraspanins include: cluster of differentiation (CD) 9, CD63, CD81, and CD82, as well as adhesion molecules CD54 and CD11b [26, 28, 78]. In addition, exosomes are enriched with heat shock proteins (hsps) which act as chaperones and play a key role in cellular responses that are associated with environmental stress. Hsps assist with protein folding and trafficking. Common exosomal proteins include Hsp60, Hsp70, Hsp90, and heat shock protein cognate 70 [16, 78]. Along with tetraspanins and hsps, exosomes contain cytoplasmic proteins such as Rabs and annexins [26, 61]. These proteins promote the fusion of MVB with the cell membrane and the removal of exosomes. Clathrin, Alix, Tumor susceptibility gene 101 (TSG) 101), and ubiquitin are exosomal constituents that are involved in the biogenesis of MVBs [78]. Enzymes that makeup the composition of exosomes consist of protein kinase G (PKG), ATPase, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and enolase. Signal transduction proteins, ADP-ribosylation factor (ARF) 1, cell division control protein 42 (CDC42), epidermal growth factor receptor (EGFR), β-catenin, guanine nucleotide-binding G proteins (G proteins), phosphatidylinositol 3-kinase (P13K), mucin 1 (MUC 1), 14-3-3, and syntenin [68, 78, 80]. Viral proteins, such as group-specific antigen (Gag), Human immunodeficiency virus negative regulatory factor (HIV Nef), Herpes simplex virus glycoprotein B

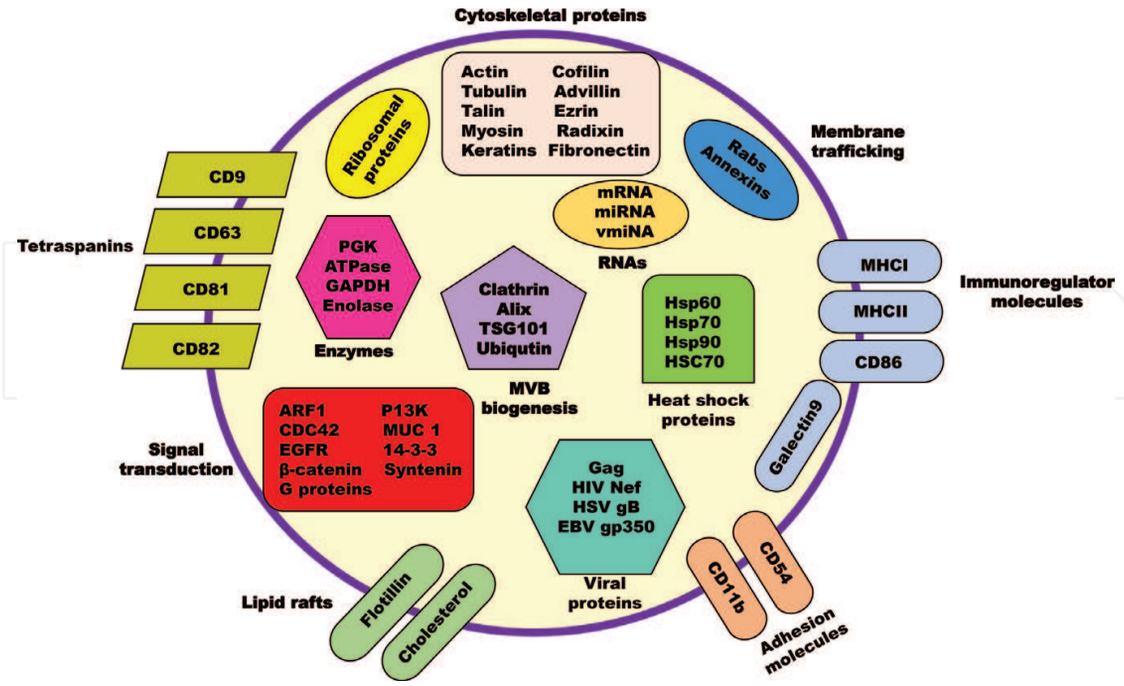

Figure 2. Composition of exosomes. Proteomic, Biochemical, and Immunological investigations have identified many specific proteins and RNAs present in some exosomes. This is a limited representation of common molecules present within some exosomes. Molecules illustrated here are grouped based on category function or protein class: Tetraspanins, Cytoskeletal proteins, Membrane trafficking proteins, Immunoregulator molecules, Adhesion molecules, Lipid rafts, Signal transduction molecules, viral proteins and RNAs.

(HSV gB), Epstein–Barr virus (EBV) gp350 also make up the composition of some exosomes [78, 81]. Exosomes that contain RNA can serve as an alternate pathway of cellular communication [82, 83]. Thus, mRNAs that are found in exosomes can be transferred to target cells and translated into proteins [84]. miRNAs, such as miR-1, miR-15, miR-16, miR-151, miR-375, and lethal-7 play a role in hematopoiesis, exocytosis, tumorigenesis, and angiogenesis [28, 85, 86].

4. Exosome isolation/purification methods

Exosomes are isolated from a wide spectrum of biological fluids [87, 88]. To examine the quality of isolated exosomes, numerous methods have been developed to examine and measure their morphology, composition, quantity, and size distribution [88, 89]. With advances in science and technology, many innovative techniques have been established to exploit a specific trait of exosomes, such as their size, shape, density, and surface proteins to aid in their isolation and purification [88, 90]. However, each method has advantages and disadvantages as shown in **Table 1**.

4.1. Ultracentrifugation and filtration-based exosome isolation

Ultracentrifugation is a centrifugation process used for generating acceleration up to 100,000 × g (approx. 9800 km/s^2) [88]. Differential ultracentrifugation is often used to isolate exosomes [88, 91]. The isolation of exosomes by differential ultracentrifugation contains numerous centrifugation steps, which uses centrifugal force to get rid of residual cells, cellular

Isolation/purification methods	Mechanism	Advantages	Disadvantages
Differential ultracentrifugation [79, 91]	Remove residual cells, large vesicles, and cellular debris; precipitate exosomes [79]	Standard method used to isolate exosomes from cultured media and biological fluids [79, 91]	Effectiveness of the method is lower when biological fluids are used for analysis [79] co-precipitation of protein aggregates, apoptotic bodies, or nucleosomal fragments, which may lead to less sample purity and less correctly bound proteins [91]
Sucrose gradient centrifugation [91]	Separate vesicles based on their different flotation densities [91, 97]	Allows separation of the low-density exosomes from other vesicles, particles and contaminants [91]	Cannot separate exosomes from viruses because of their similarities in density and size [91]
Filtration [79, 91]	Used to separate exosomes from proteins and other macroparticles using ultrafiltration membranes [79]	Allows separation of soluble molecules and small particles from exosomes [79]	Loss of analysis due to adhesion. Contamination of isolated EVs. Exosomes can potentially be deformed or damaged due to additional force being applied pass the analyzed liquid through the membrane [79, 91]
Size exclusion chromatography [79]	Applies a column packed with porous polymeric beads which separates the particles based on their size [79]	Allows precise separation of large and small molecules and application of various solutions. Compared to centrifugation methods, the structure of exosomes isolated by chromatography is not affected by shearing force [79]	Requires a long running time, which limits applications of chromatographical isolation for processing multiple biological samples [79]
Microfluidics [91]	Microscale isolation based on a variety of properties of exosomes like immunoaffinity, size, and density [91]	Energy efficient, portable, fast processing time, low cost, easy automation and integration [91]	Lack of standardization and large scale tests on clinical samples, lack of method validation, moderate to low sample capacity [91]
ExoQuick™ [91, 111]	Precipitates exosomes overnight through incubation [91, 111]	Fast and easy processing; additional equipment is not needed for isolation [91, 111]	Lack specificity toward exosomes; biological fluids are difficult to resuspend [91, 111]

Table 1. Advantages and disadvantages of isolation/purification methods.

debris, and large vesicles [79, 88]. In addition, these steps are used to precipitate exosomes [79, 88]. There are various protocols available for this isolation technique. First, cell culture is subjected to a low speed centrifugation using a Sorvall RT600 centrifuge with a swinging bucket rotor (Thermo Fisher Scientific). This is applied to remove cells and apoptotic debris [92, 93]. Next, a higher speed is used to administer and eliminate larger vesicles, whereas,

the remaining media is re-suspended in phosphate buffered saline. Lastly, a high speed of centrifugation using a SW41T1 swinging rotor in a Beckman Coulter (Brea, CA, USA) (Optima L-70 K ultracentrifuge) is performed to precipitate exosomes; and the exosome pellet is stored at −80°C until further use [92].

Filtration is a size-based technique that is often used in combination with ultracentrifugation, as depicted in **Figure 3**, for the isolation of exosomes in *in vitro* studies [92–94]. Depending on the size of vesicles, filtration is applied to separate exosomes from proteins and other particles [79]. Filtration membranes that have pore sizes of 0.22, 0.45, or 0.8 μm can be used to collect EVs that are larger than 150 nm [79]. Although filtration is a quick isolation method, it faces challenges, such as contamination of isolated EVs, trapping of EVs in nano or micro pores, and co-purifying abundant proteins EVs isolation [91]. Because of these disadvantages, the maximal recovery of EVs for isolation must be optimized [91].

4.2. Sucrose density gradient centrifugation

Sucrose density gradient centrifugation is a form of centrifugation that is used to measure the density of exosomes in a sucrose gradient [95]. Exosomes have floatation densities ranging from 1.08 to 1.22 g/ml on continuous sucrose gradients [91, 96, 97]. Vesicles that are purified from the Golgi float at 1.05 to 1.12 g/ml; and vesicles that are purified from the endoplasmic reticulum float at 1.18 to 1.25 g/ml [95]. Sucrose density gradient is formed by overlapping lower concentrations of sucrose on higher concentrations in a centrifuge tube. For instance, a sucrose gradient may contain layers ranging from 70% sucrose to 20% sucrose in 10% increments [91, 96, 97]. Since exosomes are generally spread among 3 to 5 segments of the sucrose gradient, it is recommended to perform this separation approximately 5 times the amount of exosomal proteins that is needed to detect exosomes.

4.3. Size exclusion chromatography

Size exclusion chromatography (SEC) is used to separate macroparticles based on size, not molecular weight [79]. Currently, SEC is used to isolate exosomes that are present in urine [98] and blood [79, 99]. This method utilizes a column packed with porous polymeric beads

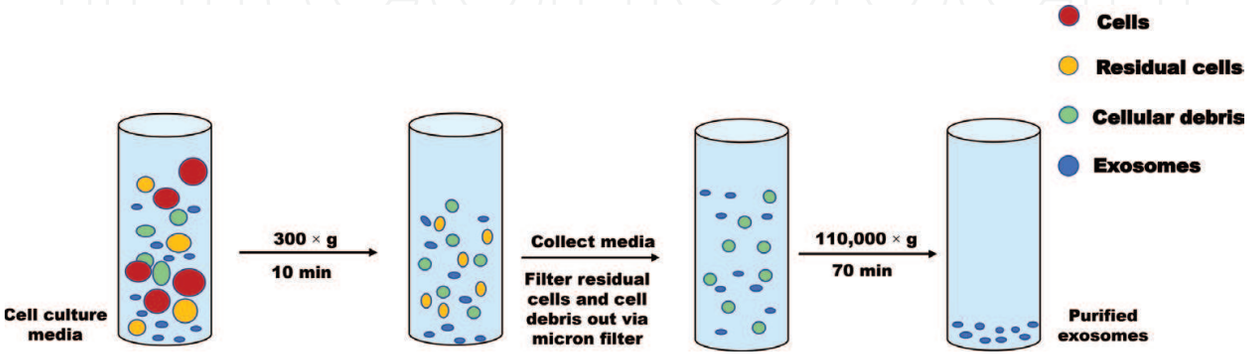

Figure 3. Schematic illustration of differential ultracentrifugation. Cell culture media is collected and centrifuged by means of low speed centrifugation followed by collection of media and filtration using a 0.22-micron filter. The media then undergoes ultracentrifugation pelleting the exosomes which are resuspended in buffer for further use.

that contains several pores and tunnels. In SEC, particles pass through the beads depending on their diameter. Particles that contain small hydrodynamic radii can pass through the pores, hence resulting in late elution [88]. However, particles that contain large hydrodynamic radii, are excluded from entering the pores [88, 100].Correspondingly, SEC is used in combination with ultracentrifugation to isolate/purify exosomes [88, 101]. Rood et al. 2010 demonstrated that ultracentrifugation followed by SEC, significantly enriched urinary exosomes compared to exosomes that were obtained by ultrafiltration or ultracentrifugation alone [101, 102].

4.4. Microfluidics

Microfluidics is the study and manipulation of fluids at the microscale level by means of frictional forces [91, 103]. Microfluidic devices bind specific EVs to antibody-coated surfaces [104, 105]. The EV sample is loaded on a pump that slowly pushes the fluid through the chip. Microfluidic-based technologies ensure that fluid pressure is converted to high shear forces more consistent and efficient than other technologies. By maintaining constant pressure, microfluidic homogenizers ensure that the samples receive the same treatment. As fluids are forced at controlled temperatures and constant pressures through the interaction chamber, particles

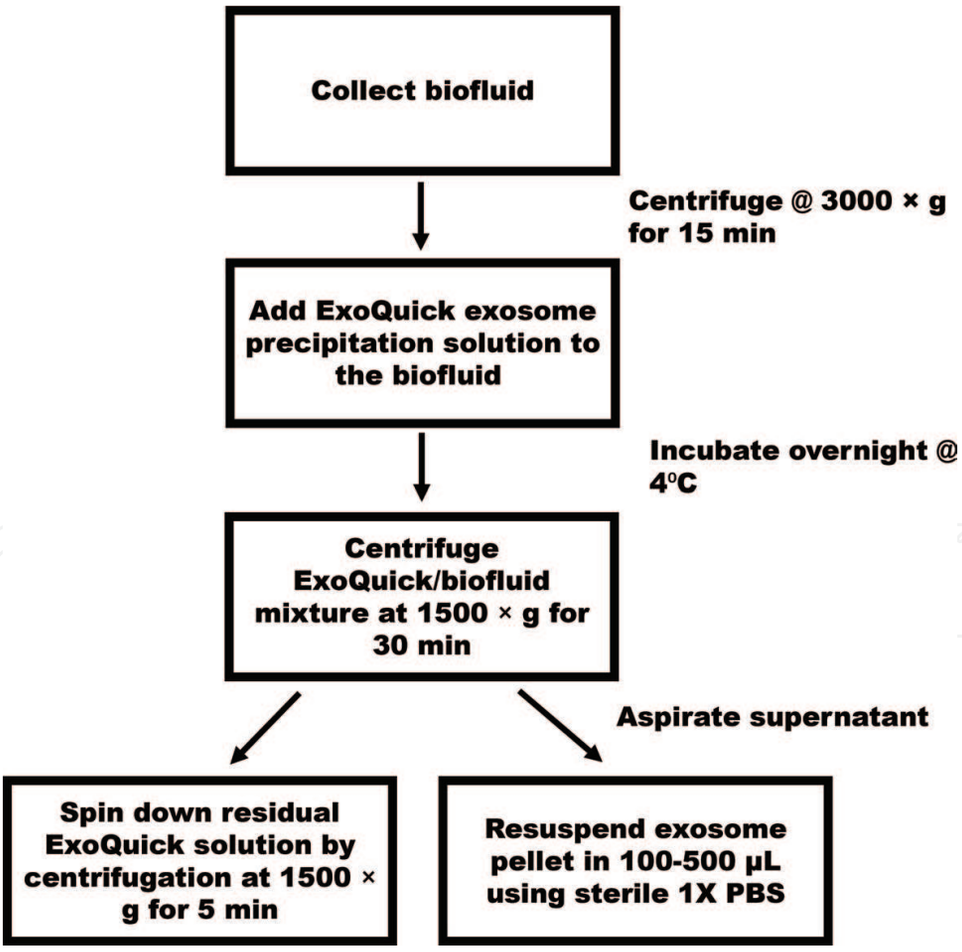

Figure 4. Standard ExoQuick™ protocol. Exosomes are collected be means of several centrifugation and precipitation steps.

experience extremely high shear forces. As a result of these forces, particle size is reduced and particle size distribution curves are constricted. Advantages of this technique include a reduction in processing times, energy consumption, volumes of sample, and material costs [106, 107].

4.5. ExoQuick™

Commercial kits utilizing polyethylene glycol for isolation of exosomes are frequently used in research studies [108–110]. ExoQuick™ (System Biosciences, Mountain View, CA, USA) is the most commonly used kit [108]. This kit is quick and easy to perform, and additional equipment is not necessary for isolation. ExoQuick is a proprietary polymer that can be used to isolate exosomes for a variety of applications, including functional studies (i.e. cell-to-cell signaling), exosomal proteomics, biomarker studies, biology studies (i.e. tumorigenesis), exosomal miRNA profiling, and exosomal metabolomics/lipidomics [111–113]. With the ExoQuick, a mix is added to the samples and the EVs precipitate via incubation overnight. Recent studies have revealed that the highest yield of exosomes was obtained using a combination of ExoQuick with ultracentrifugation [91, 114, 115]. However, contamination of exosomal isolates with non-exosomal materials remains a concern for polymer-based isolation procedures. Furthermore, the polymer substance that is present in the isolate may affect the down-stream analysis [79]. A detailed protocol utilizing ExoQuick is depicted in **Figure 4**.

5. Exosome characterization methods

There are several common techniques that are used to determine the quantity, morphology, and size of exosomes following purification. Exosomes can be characterized using the following techniques: flow cytometry [116], electron microscopy (EM) [117], NanoSight tracking analysis (NTA) [92, 117, 118], Raman spectroscopy (RS) [119], Western blot (WB) [42, 92, 120], and/or ExoCarta database [117, 121].

Flow cytometry is one of the most commonly used techniques used to detect the origin, size, and morphology of circulating EVs [116, 121]. It is a high-throughput, multi-parametric technique that quickly analyzes and quantitates thousands of single cells or particles [121, 122]. In this method, a laser beam with a specific wavelength is directed through a stream of a sheath fluid that contains suspended particles [117]. Next, the emitted scatter and fluorescence is captured and measured by detectors [121]. Due to their small diameter (≤200 nm), detecting, capturing, and examining exosomes is difficult to characterize via flow cytometry [123]. However, proteins that are located on the surface of exosomes can be stained with fluorochrome-conjugated antibodies [124].

EM is often used to characterize and visualize exosomes due their small size [117, 120]. EM uses a beam of electrons to generate an image of the EVs' sample [117]. Electron beams are passed through the sample [117]. The electrons are collected and magnified using special lenses [117]. Typical morphological characteristics of exosomes are spherical shaped and range approximately 30–100 nm [120]. When used in conjunction with immuno-labeling, the surface proteins of exosomes can be determined via electron microscopy [125].

NTA measures the concentration and size distribution of exosomes [92, 117, 126, 127]. An NTA device is composed of a laser light scattering microscope connected to a sensitive charge-coupled device camera, a complementary metal–oxide–semiconductor camera, a hydraulic pump, a measuring chamber, and an analytical software [117, 121]. The hydraulic pump injects particles into the measuring chamber at a fixed flow rate and exposes them to a narrow laser beam [117]. Next, the movement of the illuminated particles is recorded by the complementary metal-oxide-semiconductor [117]. The NTA software then identifies and tracks individual ECVs moving under Brownian motion and relates the movement to a particle size based on the Stokes-Einstein equation [128]:

$$(x, y)^2 = 2k^B T/3 r_h \pi\eta \quad (1)$$

Figure 5 depicts a graphic representation of the NTA.

RS is a quantitative technique that provides the chemical structure of exosomes based on the illumination of analyzed samples by laser light [129]. It is used to study rotational, vibrational, and other low-frequency transitions in a system [130]. Thus, it provides molecular fingerprints of the samples and enables monitoring of changes that occur in the molecular bond structures [117]. In this method, photons interact with other photons, molecular vibrations, and other excitations in the system. This interaction leads to a slight up or down shift of their energy. The shift in energy provides information about the vibrational transitions in the molecules [121, 130, 131]. Because of these measurements, the chemical composition of single EVs can be obtained [117, 131, 132].

WB is often used to show and confirm the presence of exosomal proteins and specific surface markers [133–135]. Specific surface markers include MHC I and MHC II, tetraspanins CD9, CD63, CD81, Hsp70 and Hsp90, etc. After EVs are isolated, they are lysed. Following lysis, the proteins are separated and analyzed [133]. Although WB is used to identify and confirm the presence of exosomal proteins, it cannot determine the presence of EVs alone. However, WB can be used to identify proteins in purified exosomal samples [120, 133].

Also, to help investigators validate and/or characterize their findings related to exosomes, researchers can use ExoCarta (http://www.exocarta.org/) [136]. ExoCarta is an online database that allows principal investigators the ability to identify and characterize exosomal cargos. The database contains detailed information about lipids, proteins, and RNA sequences that have been identified in specific exosomal preparations [77].

There are many other methods/techniques that are used to detect, identify, visualize, and characterize EVs. Additional techniques that have been used include: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) [137], Bradford assay [133], Enzyme-linked immunosorbent assay (ELISA) [133], dynamic light scattering (DLS) [117], mass spectrometry [137, 138], atomic force microscopy (AFM) [139], field-flow fractionation [140], and resistive pulse sensing [141]. Briefly, SDS PAGE, the Bradford assay, and ELISA are used to validate the presence of proteins. In this context, these assays could be used to confirm proteins on exosomes and/or proteins located within exosomes. Whereas, mass spectrometry, AFM, field-flow fractionation, and sensitive pulse sensing is to observe the molecular and physiochemical properties of EVs. These assays are often used to examine,

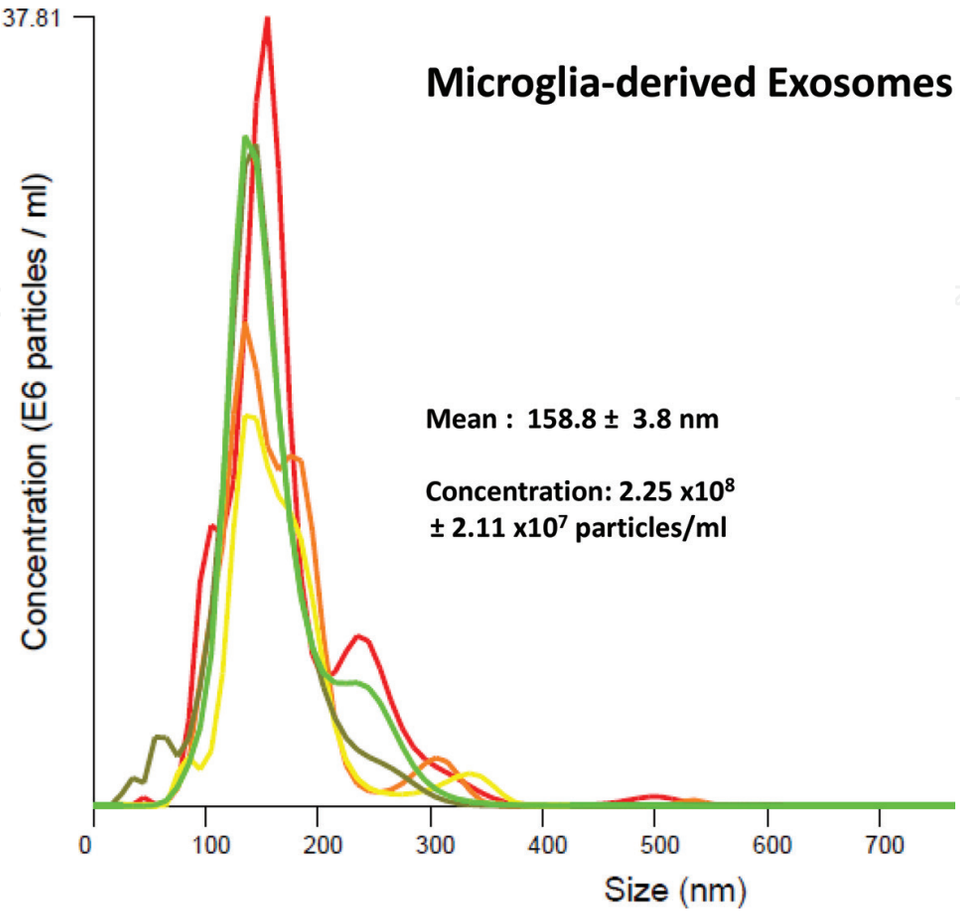

Figure 5. Representation of exosomes by NanoSight tracking analysis (NTA). Microglia-derived exosomes were generated as described in **Figure 3** and confirmed by NTA. In brief, we used the NanoSight LM10 (Malvern Instruments, Inc., Malvern, UK) and NTA v2.0 software to characterize mouse microglia-derived exosomes. All data were collected using five frames and in triplicate. Samples were diluted 1:1000 prior to tracking.

identify, and determine particle size and particle size distribution. Whenever applicable, statistical analyses should be performed to check results found from all methodologies. These analyses could include but are not limited to, Student T-test and analysis of variance (ANOVA).

6. Exosomes in cancer

Exosomes released from cancer cells may impact the cancer microenvironment significantly and alter the fate of proximal cells. These exosomes can mediate intracellular communication between other cancer cells, neighboring stromal cells, and immune cells [142–144]. In 2014, Boelens and team revealed that exosomes can be transferred from stromal cells to breast cancer cells [144, 145]. Due to this transfer, the antiviral retinoic acid-inducible gene 1 enzyme signaling can be activated to regulate the development of therapy-resistance tumor-initiating cells [144, 145]. Several studies have reported that cancer-derived exosomes play a major role in drug resistance, metastasis, angiogenesis, tumorigenesis, tumor growth, and tumor immune escape, as depicted in **Figure 6** [144, 146, 147].

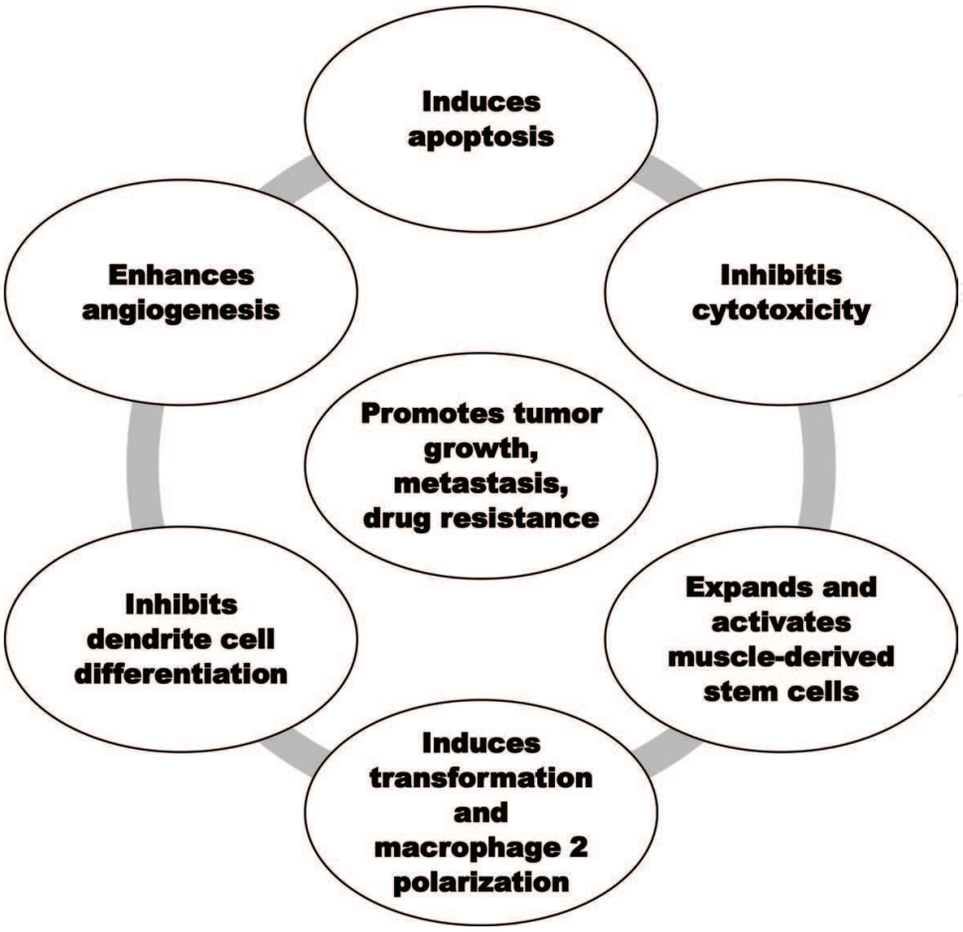

Figure 6. Roles of exosomes in cancer. The multifaceted role of exosomes in carcinogenesis.

6.1. Drug resistance

Resistances to chemotherapy, radiation, or targeted therapies are significant challenges in the treatment of various cancers [148–150]. Recently, it has been demonstrated that exosomes aid in resistance through the transfer of lipids, proteins, mRNAs, and miRNAs [80, 151], which can influence the response to anticancer drugs [152]. Corcoran et al. [153] evaluated the enhancement of exosome secretion by Docetaxel-resistance in prostate cancer. They observed that this enhancement of exosome secretion is due to docetaxel efflux through exosomes [153]. Akao et al. [154] observed that the secretion of tumor-suppressors miRs-145 and miR-34 via exosomes increased 5-fluorouracil resistance in colon cancer cells.

6.2. Metastasis, angiogenesis, and tumorigenesis

It is believed that exosomes mediate signaling in cancer metastasis [155–158]. Exosomes can function as escape routes for miRNAs and proteins that serve as promoters of metastatic pathways. The uptake of exosomes by endothelial cells can stimulate angiogenesis [144, 159, 160]. Grange et al. [159] investigated the role of angiogenesis in renal cell-derived exosomes in lung cancer ascites. Exosomes that are stimulated by hypoxia and heparanase- an enzyme that acts at the cell surface and within the extracellular matrix to degrade heparan sulfate molecules, are associated with angiogenesis of breast cancer, which is the most significant

part of breast cancer tumorigenesis [145, 149]. Tumorigenesis is the process that occurs when normal healthy cells transform into cancerous cells. During this process, these cells can secrete exosomes [146, 147]. Several studies have reported that neoplastic transformation of adipose-derived stem cells was induced in response to prostate cancer-derived exosomes [146, 161]. Also, studies have reported that these exosomes deliver mRNA molecules and oncogenic proteins to recipient cells; which subsequently induced tumor formation [146, 162].

6.3. Tumor growth and tumor immune escape

Tumor cells secrete many exosomes [163–166]. Supporting evidence has shown that exosomes released from tumors promote the formation of tumor blood vessels that support tumor growth and extension [144, 146, 149]. Glioblastoma multiforme cell-derived exosomes have been proposed to promote tumor growth by transporting RNA into recipient cells in the microenvironment [142, 143]. In 2011, Kogure and colleagues showed that hepatocellular carcinoma-derived exosomes can modify the transforming growth factor-β-activated kinase 1 expression and associated signaling pathways to augment cell growth in recipient cells [144, 167].

Cancer cells utilize exosomes that contain proteins and nucleic acids to enact an immune escape [144]. It has been shown that they activate dendritic cells, thus priming the immune system to identify and kill cancer cells [168]. Remarkably, exosomes secreted by cancer cells have been proven to express tumor antigens, as well as immune suppressive molecules, such as Fas ligand and Programmed death-ligand 1 [169]. Taken together, these data suggest that cancer cells use exosomes to further advancement of its tumorigenesis.

7. Exosomes in genetic-related diseases

Many types of cells (i.e. neurons, astrocytes, oligodendrocytes, glial) in the central nervous system secret exosomes [32, 170–172]. Exosomes have been reported to aid in the spread of pathological proteins that are involved in neurodegenerative diseases, such as Alzheimer disease (AD) [170, 173], Parkinson's disease (PD) [171, 173, 174] prion diseases [32, 173] and Huntington's disease (HD) [32]. Current studies have shown that exosomes can spread pathological misfolded proteins, which leads to the onset and propagation of AD [170, 173]. AD is the most common form of dementia and characterized by amyloid plaques and neurofibrillary tangles [170, 173]. Accumulating evidence has demonstrated that exosomes play a controversial role in the pathogenesis of Alzheimer [170, 173, 175]. Yuyama et al. [176] observed the presence of exosome-associated amyloid-ß peptide in the cerebrospinal fluid of cynomolgus monkeys and amyloid precursor protein transgenic mice. They concluded that these findings could potentially contribute to AD pathogenesis [176].

Many studies have revealed that exosomes derived from the central nervous system occur in the cerebrospinal fluid and peripheral body fluids, and their contents are altered during disease, making them an appealing target for biomarker development in PD [171, 174]. PD is a disorder that occurs due to the loss of dopamine produced in the brain affecting movement of the body [172, 177]. Exosomes may aid in the spread of toxic α-synuclein protein between

cells and induce apoptosis, which could potentially be proposed as a key mechanism underlying the spread of α-synuclein aggregates in the brain and the acceleration of pathology in PD [171, 178]. Comparative studies have shown that the expression of the PD-associated protein α-synuclein is targeted by miR-7 and miR-153 [179–181].

Prion diseases also known as transmissible spongiform encephalopathies are a group of infectious neurodegenerative disorders that affects animals and humans [172, 182]. These diseases are caused by abnormally shaped proteins called prions [177]. Exosome-mediated propagation in prion diseases was reported in 2004 by Fevrier et al. [183, 184]. They observed that the prion protein (PrP)-expressing cells could release normal PrP^{C} and abnormal PrP^{Sc} in association with exosomes [183, 184]. The first reported *in vivo* study related to prion disease pathogenesis was demonstrated 4 years later by Vella et al. 2008 [183, 185]. They revealed PrPC was associated with extracellular vesicles that were found in the CSF of sheep [183, 185].

HD is a hereditary neurodegenerative disorder that causes progressive degeneration of nerve cells in the brain due the aggregation of the mutant Huntingtin protein [186–188]. Lee et al. [186] investigated the therapeutic role of exosomes from adipose-derived stem cells by examining pathological phenotypes of a HD model *in vitro*. They confirmed that adipose stem cell-derived exosomes up-regulates the peroxisome proliferator-activated receptor gamma coactivator 1, phosphorylated cyclic AMP response element binding protein, and ameliorates abnormal apoptotic protein level in an *in vitro* HD model [186]. A year later, Soon-Tae Lee and colleagues developed an therapeutic exosome-based delivery method to treat HD using miR-124, one of the key miRNAs that is repressed in HD [189].

8. Exosomes in infectious diseases

8.1. Viruses

Exosomes derived from virus-infected cells have been shown to carry viral proteins, genetic regulatory elements, genomic RNA, mRNA, and miRNA [50, 190, 191]. Depending on the genetic material and proteins incorporated into them, EVs may play a vital role in viral infection, especially in retroviruses [192]. Retroviruses are enveloped RNA viruses that replicate through a DNA intermediate inserted in the host cell genome [193]. According to the Trojan hypothesis, it is believed that retroviruses exploit preexisting pathways for intracellular trafficking [192, 194]. Thus, the Trojan hypothesis states that retroviruses use the preexisting, nonviral exosome biogenesis pathway for the formation of infectious particles, and the preexisting, nonviral pathway of exosome uptake for a receptor-independent, enveloped-independent mode of infection [81, 194–196].

Among the retroviruses, HIV-1 is the most common studied virus [127, 197]. Exosomes isolated from patients with HIV infection or from HIV-1 infected cells incorporate the viral transactivating response element that is transcribed from the integrated provirus [50, 198]. This is believed to stimulate HIV-1 replication in recipient cells by downregulation of apoptosis [50, 197–199]. Madison et al. [200] showed that semen-derived exosomes inhibit HIV-1 replication

in various cell types. Years later, Madison and colleagues described detailed protocols for evaluating the function and physical properties of these semen-derived exosomes [200] for *in vitro* uptake and HIV-1 infection assays [201]. Recently, Sims et al. [92] have demonstrated the role of T cell immunoglobulin and mucin proteins (TIM) in exosome-dependent HIV-1 trafficking into human immune cells. Through viral infection assays, they demonstrated that exosomes derived from human lung carcinoma, human breast milk, human plasma, and mouse neural stem cells, increased HIV-1 entry into macrophages and T cells [92]. Furthermore, they demonstrated that HIV-1 and exosome interactions were potentially mediated through binding of TIM4 to the viral envelope [92]. In another study, Sims and colleagues demonstrated that exosomes can enhance HIV-1 entry into human monocytic and T cell lines through exosomal tetraspanin proteins CD9 and CD81 [127].

8.2. Bacteria-derived exosomes

Bacteria make and release membranous vesicles [202–204]. Gram-negative bacteria produce outer-membrane vesicles that originate from the blebbing of the outer membrane [202]. Also, they form vesicles that contain membrane components, nucleic acids, and proteins [202]. Many gram-negative bacteria that produce these vesicles are pathogenic and toxic to host cells [202, 205–207]. However, they can deliver antigens; and therefore, act as a potential vaccine candidate [202, 206, 207].

Gram-positive bacteria produce outer-membrane vesicles [202, 208]. Unlike gram-negative bacteria, these vesicles play a role in inter-species and intra-species communications [202, 209], in addition to potential inter-kingdom interaction with the host [202, 206, 210]. Most importantly, these vesicles provide an innovative approach for development of non-live vaccines [202]. These vaccines have been successfully used with children infected with *Neisseria meningitidis* in New Zealand [211].

8.3. Parasitic-derived exosomes

There is accumulating evidence that has reported the release of EVs in parasitic diseases, acting in parasite–parasite inter-communication and in parasite-host interactions [212–214]. EVs participate in the dissemination of the pathogen and play a vital role in host-pathogen interactions [212, 215]. Vesicles that are secreted by infected cells contain large amounts of pathogen molecules, which are sufficient to induce modifications in non-infected neighboring cells or act as antigen presenters for the immune system [215]. In 2013, Hassani and Olivier identified GP63 surface protease of *Leishmania mexicana* on exosomes [202, 216]. They observed that this protease could be transmitted to distant sites by enzymatic activity [202, 216].

9. Exosomes as diagnostic and therapeutic biomarkers

Exosomes have attracted enormous research interest because of their promising medical applications [217–219]. Exosomes may serve as diagnostic tools because they are carriers

of molecular markers of many diseases and as a prospective delivery system for various therapeutic agents [75, 220–222]. Supporting evidence suggests that exosomes are present in all bodily fluids and may be associated with disease pathogenesis [223–226] and may be involved in cellular protection [227, 228]. Mostly importantly, they contain various nucleic acids, lipids, and proteins. Due to the cargo of exosomes, exosomes are involved in several infectious diseases [75]. Because of their endocytic origin, exosomes carry specialized protein markers, such as hsps, tetraspanin, and Rab family proteins. Exosomal content is a fingerprint of the state (cancer versus quiescent) of the cell and the original cell type.

Exosomes can be used to diagnosis various diseases, such as cancer, AD, PD, HD, etc. [150, 173, 174, 186, 229]. Non-invasive diagnostics (using saliva and urine samples) or minimum invasive diagnostics (based on blood analysis) make exosomes very attractive alternatives to excision biopsies or traditional needle biopsies. There are advantages such as lower cost analysis, convenience and reduction in patient pain [229].

Exosomes can be exploited as potential carriers for therapeutics [230]. Many anti-inflammatory drugs (i.e. Doxorubicin) can be inserted into purified exosomes for *in vivo* and *in vitro* applications [231–235]. Sun et al. [236] investigated the anti-inflammatory activities of curcumin when encapsulated in exosomes. A year later, Zhuang and colleagues demonstrated that exosomes can be utilized to deliver anti-inflammatory drugs to the brain through a non-invasive intranasal route [232].

10. Summary

Secreted exosomes have important functions in the pathogenesis of various diseases. Several methods have been developed to isolate, purify, and characterize exosomes from biological fluids. However, isolation of exosomes can be problematic during the purification process due contaminants, such as protein aggregates, microvesicles, microbes, etc. Because of theses contaminates, it is challenging to characterize exosomes accurately, and use them for experimental assays. Centrifugation techniques remain very common. However, other methods, such as filtration, sucrose density gradient centrifugation, SEC, microfluidics, and ExoQuick™ show promising results and can be effectively applied both in laboratory research and clinical medicine. It is most important to note that subsequent to exosome purification it is necessary to employ a combination of methods to confirm and characterize extracellular vesicles. The utilization of multimodality validations will allow researchers to obtain data that is qualitative, quantitative or both. Characterization of exosomes allows researchers to understand exosomal properties and function. Most importantly, characterization studies allow researchers to identify unique exosomal marker proteins to detect the presence of exosomes found in cell culture supernatants and biological fluids. The study of EV composition has shown that they can carry numerous cargos (i.e. lipids, proteins, and nucleic acids). These cargos can vary widely between cells and conditions. Their composition is cell type-dependent that can be altered by different environmental factors. The use of exosomes as therapeutic delivery vehicles covers a wide array of diseases, including but not limited to cancer, virus-induced diseases, genetically related diseases and parasitic diseases.

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