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Role of Exosomes for Delivery of Chemotherapeutic Drugs

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ABSTRACT: Exosomes are endogenous extracellular vesicles (30–100 nm) composed with membrane lipid bilayer which carry vesicular proteins, enzymes, mRNA, miRNA and nucleic acids. They act as messengers for intra- and inter-cellular communication. In addition to their physiological roles, exosomes have the potential to encapsulate and deliver small chemotherapeutic drugs and biological molecules such as proteins and nucleic acid-based drugs to the recipient tissue or organs. Due to their biological properties, exosomes have better organotropism, homing capacity, cellular uptake and cargo release ability than other synthetic nano-drug carriers such as liposomes, micelles and nanogels. The secretion of tumor-derived exosomes is increased in the hypoxic and acidic tumor microenvironment, which can be used as a target for nontoxic and nonimmunogenic drug delivery vehicles for various cancers. Moreover, exosomes have the potential to carry both hydrophilic and hydrophobic chemotherapeutic drugs, bypass RES effect and bypass BBB. Exosomes can be isolated from other types of EVs and cell debris based on their size, density and specific surface proteins through ultracentrifugation, density gradient separation, precipitation, immunoaffinity interaction and gel filtration. Drugs can be loaded into exosomes at the biogenesis stage or with the isolated exosomes by incubation, electroporation, extrusion or sonication methods. Finally, exosomal cargo vehicles can be characterized by ultrastructural microscopic analysis. In this review we intend to summarize the inception, structure and function of the exosomes, role of exosomes in immunological regulation and cancer, methods of isolation and characterization of exosomes and products under clinical trials. This review will provide an inclusive insight of exosomes in drug delivery.

KEY WORDS: exosomes, extracellular vesicles, biogenesis, tumor microenvironment, drug delivery, nano drug carriers

I. INTRODUCTION

Tumour microenvironment (TME) have several barriers to deliver therapeutic drugs into the site of action. Vascular endothelial boundaries, mononuclear phagocyte framework, low pH, low oxygenation and high interstitial liquid weight are the most widely recognized TME obstructions.[1,2] It is a big challenge to deliver a sufficient quantity of chemotherapeutic drugs to the target tumor site without harmful side effects to the normal tissue.[3] Nano drug delivery is one of the strategies which has the potential benefit

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to overcome the challenge related to chemotherapeutic drug failure and off-target toxicity.[4–11] Nano drug carriers (NDCs) increase drug solubility, bioavailability and biodistribution.[12,13] Moreover, passively targeted NDCs act via enhanced permeability and retention effect by passing through leaky vasculature and poor lymphatic drainage of tumor site.[14–17]

Liposomes, micelles, dendrimers, antibodies and polymeric materials are among the recently emerged and clinically approved NDCs.[18–23] Currently, there are various, clinically approved nanotechnology-based chemotherapeutic drugs. There are also numerous drugs at clinical and preclinical stages of development.[24] NDCs can encapsulate ionic, lipophilic and hydrophilic as well as hydrophobic drugs. Ideal NDCs should be stable, nonimmunogenic, biodegradable, cost-effective, easy to fabricate, convenient for drug loading and be able to release their cargo at the targeted site of action.[25–27] However, opsonization of nanoparticles by the reticuloendothelial system (RES) is the main drawback of NDCs.[28] Modification of NDCs with polyethylene glycol (PEG) serves to by-pass the RES and improves the flow half-existence of medications and subsequently helps to deliver at targeted areas with increased restorative adequacy.[29,30] It is still challenging to develop an ideal NDCs by formulation driven approach.[31] Currently, there is an upcoming new paradigm to use exosome vehicle in place of synthetic lipidic or polymeric NDCs.[32–36] Exosomes, a type of extracellular vesicles (EVs) which can be secreted naturally by all kinds of cells and can be readily found in different body fluids.[37,38] Like liposomes, exosomes are also a phospholipid bilayer containing cell-derived vesicles which can load different proteins, lipids and nucleic acids.[39] Unlike other synthetic NDCs vesicles, exosomes are composed of varying surface membrane proteins.[39] Exosomes are 30–150 nm in diameter, with vesicular membrane thickness of 5–8 nm, which carry a net negative charge with a density range between 1.13–1.19 g/mL.[40,41] Exosomes have zeta potential in the range of –10 to –50 mV in phosphate buffered saline.[42,43]

Endogenous NDCs can deliver chemotherapeutic drugs with higher efficiency and lesser side effects than synthetic NDCs.[44,45] Exosomes have a described natural ability to transport functional biomolecules, such as RNAs, DNAs, and proteins in their lumen. Naturally, exosomes have also favorable tumor homing property and high stability. These properties have enabled them to be considered as future suitable NDCs for chemotherapeutics.[46,47] In this review, we delineate the latest studies on the role of exosomes as endogenous NDCs for chemotherapeutic agents. Further, background information on the biogenesis, biological function, isolation mechanisms, characterization methods, drug loading mechanisms, and the exosomes under clinical trials has also been discussed.

II. BIOGENESIS, CARGO SORTING, EXOCYTOSIS, CELLULAR UPTAKE, AND VESICULAR RELEASE OF EXOSOMES

Exosome biogenesis includes several intra-cellular processes; endocytosis of the plasma membrane, formation of early endosome (EE), transformation of EE into late endosome

(LE)/multivesicular bodies (MVBs), fusion of MVBs to plasma membrane for exosome release, or fusion of MVB to the lysosomes for degradation.[48] An overview of the biogenesis process of exosomes is represented in Fig. 1. EE are formed close to active endocytosis site through the invaginated plasma membrane to maintain endocytic space for lipids, proteins and other molecules.[49–51] The EE transform into LE or MVB. Intraluminal vesicles (ILVs) are formed from LE through inward budding of MVBs.[52,53] ILVs are packages of sorted trace cytosolic components, transmembrane proteins and nucleic acid components.[54] MVBs fuse to the plasma membrane and release ILVs into extracellular space as exosomes. In contrary, some portions of MVBs fuse to lysosomes for degradation.[54,55] However, it is currently unknown what mechanisms differentiate "secretary MVB from degradative MVB."[56]

Exosomes biogenesis is closely related to the sorting of cargo molecules.[57] Exosomes get their vesicular components through endosomal sorting complexes required for transport (ESCRT) or ESCRT independent pathways.[58,59] ESCRT complexes are cytoplasmic proteins which take part in membrane budding or bending MVB biogenesis and takes part in sorting of proteins, non-ubiquitinated, lipids and nucleic acids into the ILV.[60] Vacuolar protein sorting protein 31(VPS-31), VPS-4B and tumor susceptibility gene 101 protein (TSG101) are ESCRT proteins which sort exosomal cargo into MVBs/ILV.[61,62] ESCRT independent pathway involves melanosomes, sphingomyelinase pathway and ATP-biding cassette transporters (ABT).[48,63–66]

Melanosomes are specialized endosomal structure present in pigment cells which create MVB like morphology for ILVs formation.[63,64,67] The exosomal membranes

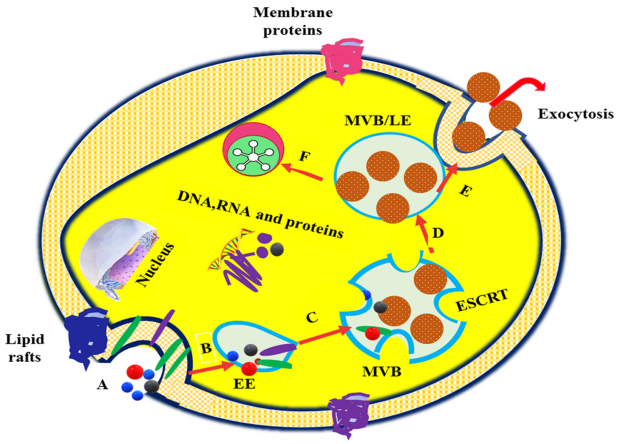

FIG. 1: Exosome biogenesis, cargo sorting, and release. Vesicle formation and cargo sorting starts from the endocytosis and ends in the MVBs. (A) Endocytosis of the plasma membrane. (B) Uptake of proteins, nucleic acids, and membrane–associated molecules into encysted body and formation of EE. (C) Transformation of EE in to MVB/LE, ILV formation by inward budding of MVB/LE, and cargo sorting through ESCRT and ESCRT independent pathways. (D) Fusion of MVB/LE with the plasma membrane and lysosome. (E) Exocytosis of exosome in response of MVB/plasma membrane interaction. (F) Degradation of MVBs/LE by lysosome.

have a lipid raft membrane with lipid raft association protein which is involved in protein sorting and exosome assembly.[65,68,69] Moreover, cell surface protein components like tetraspanin proteins (CD63) and ceramide helps to load proteins into ILVs through ESCRT independent pathways.[48]

Subsequent to exosome cargo sorting, a fusion of MVBs with the plasma membrane leads to exosome exocytosis and release to the local and distant environment.[70] The recruitment of exocytic membrane (SNAREs) to the anchored MVB is the essential step, and then small GTPases (Rab27a, Rab27b) take part in the final release role.[71–73] SNARE proteins (SNAP23, syntaxin-4 and VAMP7) are involved in the release of exosomes. However, reports by Edgar et al., Mobius et al., and Verweijet et al. demonstrated that some portion of exosomes remain attached to the parent cell surface and are involved in signaling platforms for juxtracrine communication.[74–76]

Exosomal internalization and intracellular trafficking are the vital steps to cross the biological barriers and deliver the vesicular cargo components.[77] Cellular uptake of exosomes has been shown to occur via; clathrin-mediated endocytosis, lipid raft-mediated endocytosis, heparin sulfate proteoglycans-dependent endocytosis, phagocytosis or by direct fusion with the plasma membrane.[78–81] Katrin et al. demonstrated that the uptake of exosome depends on the extracellular signal-regulated kinase, Hsp27 signaling and lipid raft mediated endocytosis.[82] The fusion of internalized exosomes with lysosomes/endosomes results in cargo release in the cytoplasm and transfer of cellular information from donor to recipient cells.[70] Figure 2 shows the multi-level process of exosome uptake, internalization and cargo release.

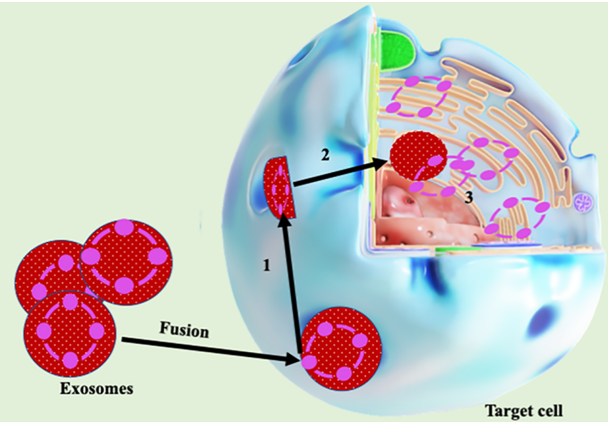

FIG. 2: Mechanism of exosome–cell interaction, cellular uptake, and cargo release. Ligands on the exosome surface membrane bind to the target cell membrane and fusion of exosome and uptake of exosome occur through: (1) Fusion of the exosomal membrane with the cellular membrane, (2) endocytosis and downstream signaling cascade, and (3) fusion of exosomes into endosome/lysosome and cargo release into the cytosol.

III. VESICULAR COMPOSITION OF EXOSOMES

Exosomes have phospholipid bilayer and contain various exosomal membrane surface proteins.The bio-synthesized exosome membranes are composed of flotillin, annexins, GTPases, sphingomyelin, cholesterol, ceramide and also MHC-II. In addition to those, endosome-specific tetraspanins and biogenesis-related proteins appear on the surface of the exosome membrane.[51,54] Tetraspanins (CD9, CD9, CD37, CD53, CD63, CD81 and CD82) are exosomal surface proteins which take part in fusion, penetration and invasion processes of exosomes.[83] Heat shock proteins (Hsp27, Hsp60, Hsp70, and Hsp90) are also packed abundantly in exosomes as part of a stress response.[83] Hsps are involved in antigen binding, presentation and even in preventing *in vitro* aggregation of unfolded proteins through hydrophobic interaction.[84] Cell adhesion proteins (integrins, lactadherin and intercellular adhesion molecule 1) are also found in exosomes which help in fusion of exosomes to cells and extracellular matrix.[85] Major histocompatibility molecules (MHC) class I and II are mainly found in mast cells (MCs) derived exosomes and these play a key role in immunoregulation.[83,86] MVB formation proteins (Tsg101, Alix, and Vps) are exosomal proteins involved in exosomes release and transport.[52] Rab family proteins (Rab2, Rab5, Rab27, Rab35 and Rab7) are also found in exosomes and are involved in exosome biogenesis, release, transport and docking of the MVB to the plasma membrane.[83,87,88]

The composition of lipids are thought to be different according to the source of exosomes.[54] However, the majority of exosomes are composed of sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserines and phosphatidylinositol.[89] These lipids have a function in vesicular membrane formation and stability of exosomes. Epidermal growth factor (EGFR) signal transduction proteins, β-catenin proteins, phosphatidylinositol 3-kinase (P13K), and mucin are also components of exosomes that likely participate in cell signaling events in recipient cells.[90,91]

Exosomes are mini-illustrative version of parental cells with major intra-vesicular conglomerations of proteins, lipids and nucleic acids.[92,93] The complexity and diversity of vesicular packages are mainly determined by their donor cells.[94,95] Based on the latest studies, there are around 4563 proteins, 194 lipids, 1639 mRNA and 764 miRNAs found in exosomes.[96–99] Figure 3 shows the schematic diagram of the exosomal surface and vesicular core components. The core of the exosomes is composed of proteins, nucleic acids and enzymes. Exosomal proteins are also miscellaneous based on the sources and parent cells.[100,101] Glycoproteins, ESCRT proteins and membrane transport proteins (associated lysosomal proteins, CD13 and PG regulatory proteins) are also components of exosomal vesicles. Other major components depending on the cell source include; histones, ribosomal proteins, ubiquitin, major vault protein and complement factor 3.[83] Proteins related to metabolic enzymes like fatty acid synthetase, glyceraldehyde-3-phosphatedehydrogenase,phosphoglyceratekinase1, ATPase, phosphoglycerate mutase 1, pyruvate kinase isozymes M1/M2, ATP citrate lyase, glucose-6-phosphate isomerase, peroxiredoxin1, aspartate

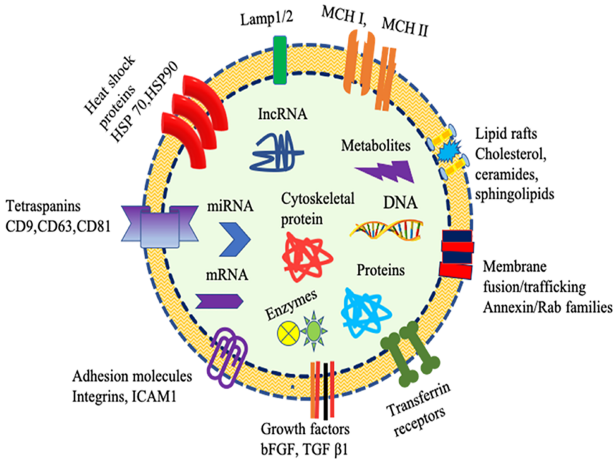

FIG. 3: Exosome vesicle and vesicular components. Exosomes have a phospholipid bilayer (yellow) and contain various exosomal membrane surface proteins. The core of the exosomes is composed of proteins, nucleic acids, and enzymes.

aminotransferase and aldehyde reductase are some of the common enzymes identified in exosomal vesicles.[83,102–104]

IV. SOURCES OF EXOSOMES

Exosomes are secreted by almost all types of endothelial and cancer cells and found in different body fluids such as blood plasma, urine, milk, saliva, amniotic, bronchioalveolar, synovial and also in ascites fluids.[105,106] Among the circulating exosomes, 80–90% are derived from platelets, lymphocytes, dendritic cells (DCs) and other immune cells. Epithelial cells, macrophages, mast cells, reticulocytes, neurons, B-cells, T-cells, oligodendrocytes, Schwann cells and tumor cells are readily studied source of exosomes.[83,107–109]

The vesicular composition of exosomes is entirely dependent on parental cells and fluids and it is an indicator for pathophysiological roles in any organisms.[110] Ferguson et al. reported the integrin signaling differences between prostate cancer cell (PC3) derived exosomes and human embryonic kidney cells (HEK) 293 derived exosomes.[110] The PC3 derived exosomes contained more mRNA than HEK. In other studies, milk-derived exosomes have been reported as immunomodulatory inducers.[111,112] The report by Agrawal et al. demonstrated that milk-derived exosomes were used for oral delivery of paclitaxel.[113] Chen et al. revealed that porcine milk-derived exosomes could promote the proliferation of intestinal epithelial cells.[114] Exosomes from biliary fluids have shown significant regulatory roles in cholangiocytes proliferation and viral inhibition.[115] Tumor-derived exosomes play a role in cell and microenvironment interactions.[116,117] Adipocytes and HepG-2 cells are also reported

as sources of exosomes and showed the interactions of cells and microenvironment communications.[118]

V. PATHOPHYSIOLOGICAL ROLES OF EXOSOMES

Exosomes intervene in cellular or organ crosstalk in both physiologic and pathologic conditions through autocrine, paracrine or endocrine signaling pathways.[118–121] Exosomes play a pivotal role in bioactive molecule transportation, immune response, antigen-presentation, protein quality regulation, cellular homeostasis and extracellular matrix remodelling.[122–125] Exosomes are involved in mRNA transfer and mRNA regulation in the recipient cells and even in protein translation.[126] So, exosomes transfer information from parent cells to the neighbor or target cells through direct stimulation by surface-bound ligands and epigenetic programming.[127]

Exosomal surface proteins and intra-vesicular components determine the specific functions of exosomes.[39] Exosomes balance brain homeostasis and nervous system plasticity by promoting the release of toxic or harmful intracellular components including damaged proteins, lipids or nucleic acids.[128,129] Exosomal protein transporters have a role in protein trafficking and also in adjusting the homeostasis of the intracellular compartment by removing obsolete and toxic components.[128,130] Exosomes serve as a mediator of communication between astroglia and neuron in the central nervous system (CNS).[131] Exosomes can also mediate the signaling between pancreatic β-cells.[132] Exosomes take part in glucose metabolism and regulation through insulin signaling cascade at the peripheral tissues.[133] Exosomes mediate the crosstalk between cardiac cells and concurrently, cardiac cell-derived exosomes can be an informative marker for homeostasis of the cardiovascular system. Exosomes derived from cardiovascular origin can also be found in blood and pericardial fluids which have a role in endocrine signaling.[134] Urinary exosomes help to detect potential biomarkers of renal disease and prostate cancers.[135,136] Exosomes from urine-derived stem cells have direct physiological role inhibiting podocyte apoptosis, promote vascular regeneration and increase cell survival by suppressing caspase-3 overexpression and increasing glomerular endothelial cell proliferation.[137] miRNAs derived from urinary exosomes serve as biomarkers for salt sensitivity and inverse salt sensitivity of hypertension pathways.[138] Urinary exosomes also transfer miRNA to tubular cells and modulate ROMK1 potassium channel level in collecting duct cells.[139,140]

Exosomes are also involved in several pathogeneses, including autoimmune disease progression, tumor metastasis and infection transmissions.[141] Exosomes transport pathological neurotic proteins between neuron cells and evolve in different neurodegenerative diseases.[142] Similarly, exosomes are also involved in insulin resistance and obesity through the insulin pathway.[143,144] Deng et al. revealed that adipose tissue-derived exosomes mediate the communication between adipose tissue and macrophages and have roles in insulin resistance through the TLR4/TRIF pathways.[145] Exosomes may accelerate or inhibit the process of bacterial, viral and malarial infections by carrying substances of pathogen origin.[146]

VI. ROLE OF EXOSOMES IN IMMUNE REGULATION

Immune regulation is among one of the biological roles of exosomes which involves immune activation, antigen expression regulation and immune suppression.[147] Shenoda et al. showed the potential of antigen-presenting cells (APCs) derived exosomes in immune response modulation.[148] DCs and macrophages derived exosomes have the role of delivering and presenting functional MHC-peptide complexes to the target cells.[149] Raposo et al. demonstrated the part of B-cell–derived exosomes in antigen presentation and T-cell activation.[150] In this study, DCs derived exosomes also expressed class I and II MHC molecules. DC derived exosomes can activate $CD8^+$ and $CD4^+$ T cells and induce an immune response.[151] Hepatocellular carcinoma (HCC) derived exosomes express significant amount of Hsp70, enhance tumor immunogenicity and increase the activity of NK cells by up regulation of the inhibitory receptor CD94 and down regulation of the activating receptor CD69.[152]

Exosomes secreted in the TME may trigger immune cells for antitumor activity or may inhibit their antitumor activity.[153] Rao et al. demonstrated that tumor cell-derived exosomes (TDE) activate the immune response and enhance the anticancer effects.[154] Conversely, tumor derived exosomes can help in tumor microenvironment programming, tumor progression, and metastasis by silencing antitumor immune response. A study by Poggio et al. showed that tumor-derived exosomes inhibit the activation of T-cell by its PD-L1 proteins and the tumor growth is thought to be suppressed by blocking exosomal PD-L1.[155]

Recently, a study by Jonathan et al., Damo et al., and others showed the potential benefit of DCs derived exosomes for cancer vaccine development.[156–162] Graeme et al. showed that exosomal Hsp70 can activate natural killer cells[163] and macrophages.[164] Akiko et al. demonstrated the role of exosomes in cellular homeostasis by removing harmful DNA from the target cells, preventing aberrant immune responses and blocking viral cellular machinery.[165]

Though, exosomes are essential compositions for viral and bacterial infection and pathogenesis, and they are also involved in an immune response against pathogens by delivering antiviral factors to neighbouring cells.[166] Platelet-derived exosomes carry prostaglandins and play a crucial role in coagulation, thrombosis, vascular senescence, permeability and also an inflammatory function.[167]

VII. ROLE OF EXOSOMES IN THE TUMOR MICROENVIRONMENT

TME is the indicative marker of cancer evolution, and it is a vital factor for tumor growth and metastasis.[168,169] TDEs share tremendous similar biological compositions with their parent cancer cells.[170] TDEs contain tumor derived DNAs, RNAs, growth factors, angiogenetic factors, extracellular matrix (ECM) molecules, enzymes, and different proteins. Exosomes have several roles in tumor evolution and progression, including tumor angiogenesis, tumor cell proliferation, migration, metastasis and apoptosis.[171–174] Corcoran et al. demonstrated the role of TDE in tumor–tumor and tumor–fibroblast communication

by transferring chemoresistance substances.[175] TDE can cause vascular leakage and dissemination of tumor cells.[176,177] TDE induces angiogenesis in TME through cargo compositions such as vascular endothelial growth factor (VEGF) and angiopoietin.[173] Xie et al. reported that human hepatocellular carcinoma secretes exosomal angiopoietin-2 and has a novel pathway to induce tumor angiogenesis.[178] Song et al. reported that TDE or small EVs promote angiogenesis by heparin bound bevacizumab-insensitive VEGF.[179] TDE can also induce epithelial-mesenchymal transition, degrade the matrix, activate macrophages and disturb endothelial cells.[174] Exosomes have a signal-mediating role between tumor cells and their surrounding cells.[180,181] TDE may help in the formation of stiff microenvironment through ECM remodeling. Exosome mediated ECM remodeling promotes local tumor invasion in the TME.[182]

In the TME, cancer-associated fibroblasts (CAF) secrete exosomes which might enhance tumor growth under nutrient deprivation conditions.[183] Tumor cells secrete exosomes in hypoxic conditions, and these TDE activate glycolysis for cancer cell hypoxic environment resistance.[184] The glycolysis process is through miRNA-145/HIF-1α/PDK1 pathway. Exosomes enhance tumor invasion, motility and metastasis by inducing the expression of TGF-β and TGF-β-activated kinase-1.[185] TDE play a rate limiting role in the modulation and shaping of TME through transferring oncogenic features to the surrounding endothelial cells.[185]

VIII. EXOSOMES AS NANO-SIZED CHEMOTHERAPEUTIC DRUG DELIVERY VEHICLES

Ideal NDCs should be able to load and convey a targeted drug to the target tissue, recognized by targeted ligand, deliver the payload at the targeted tissue and bypass the off-target toxicity of healthy cells.[186] Exosomes are thought to be a standard nano-drug carrier with numerous benefits. They are highly stable and have the ability to cross the blood–brain barrier (BBB), intrinsic ability to target specific tissues without immunogenicity problems.[14,187,188] The BBB is the main challenging factor for delivering chemotherapeutic drugs to glioblastoma (GBM) therapy. Due to this, chemotherapeutics drugs like DOX, PTX, methotrexate and vincristine demonstrated poor therapeutic effect against GBM.[201,202] Due to its physical characteristics and biological origin, exosome-based drug delivery may bring a better outcome for GBM treatment due to enhanced permeability through BBB. Yang et al. revealed that exosome-based delivery of anticancer drugs could help them cross the BBB through receptor mediated endocytosis. Brain specific exosomal surface markers like CD63 tetraspanins act as transmembrane protein, transferrin, LDL and insulin receptors help the exosome encapsulated drug to by-pass the barrier.[203] A similar study by Ruenn et al. explained the use of MSC derived exosomes as a delivery vesicle for proteasome and its potential benefit to bypass BBB.[204] A study by Ibolya et al. suggested that BBB-derived exosomes can be used as NDC and they have ability to bypass the BBB.[205] PTX and DOX encapsulated in the mouse brain endothelial cell line-derived exosomes crossed the BBB and showed better efficacy in a zebrafish model.[205] Further, due to their nano-size and endogenous property,

exosome-based chemotherapeutic delivery is thought to improve drug stability, blood circulation time, accumulation in tumor site and enhance cytotoxicity.[206]

Exosomes can shuttle small chemotherapeutic drugs to the tumor site.[207,208] Table 1 represents exosomes as potential candidates for chemotherapeutic drug delivery with different drug loading methods and sources of exosomes. The chemotherapeutic Doxorubicin (DOX), Paclitaxel (PTX) and Withaferin A can be delivered to the target site with milk-derived exosomes.[209] Additionally, bovine milk-derived exosomes showed potential benefits for oral drug delivery and prevented gastric acid degradation of a drug molecule. The exosomal delivery of those chemotherapeutics showed higher antiproliferative efficacy than free drugs against A549 lung cancer cell lines. Sun et al. showed the potential of exosome for curcumin delivery with enhanced anti-inflammatory activity compared with free curcumin.[190] *In vivo* and *in vitro* results demonstrated that the solubility, stability, and bioavailability were increased due to encapsulation of curcumin in exosomes. Schindler et al. demonstrated the exosomal delivery of DOX enables rapid cell entry and enhanced *in vitro* cytotoxicity.[210] The intracellular accumulation of exosomal DOX was improved by 18-, 21-, and 65-fold compared with free DOX, Myocet, and Doxil, respectively. The data show the potential of exosome as better endogenous cargo vehicle compared with synthetic NDC. A similar study by Gong et al. showed a nanoscale target specific exosome as the co-delivery vehicle of

TABLE 1: Drug–loading methods, exosome origin, and delivery of exosomal cargo

Methods	Exosome origin	Route	Therapeutic
Incubation Drugs with cells or drugs with isolated exosomes	MSCs (pre–loading)	*In vitro*	PTX[189]
	EL–4 cells Macrophages	Intravenous Intranasal	Curcumin[190] Curcumin[191]
	Monocytes/macrophages	*In vitro*	DOX[192]
	Endothelial//tumor	*In vitro*	Porphyrin[193]
	Glioblastoma cells	Intravenous	siRNA[194]
	Macrophages	*In vitro*	PTX[195]
Electroporation	Dendritic cells	*In vitro*	DOX[196]
	Macrophages	Intravenous/*in vitro*	PTX[195]
	Endothelial//tumor	*In vitro*	Porphyrin[193]
	DCs	Intravenous	siRNA[197]
Sonication	Macrophages	Intravenous/ *in vitro*	PTX[195]
	HEK 297 and MCF–7	*In vitro*	siRNA[198] ssDNA[199]
	Macrophages	Intranasally	Catalase[200]
Extrusion	Endothelial/tumor	*In vitro*	Porphyrin[193]
Freeze and thaw cycles	Monocyte/macrophage	*In vitro*	Catalase/ DOX[192,193]

DOX and cholesterol-modified miRNA to triple-negative breast cancer (TNBC) cells.[211] Disintegrin and metalloproteinase 15 (A15) were used as targeting ligands for macrophage-derived exosome for the co-delivery in TNBC treatment.[212,213]

Besides small chemotherapeutic drugs, exosomes show good cargo versatility to deliver small nucleic acid and proteins as well. Exosomes have a natural capacity to transport siRNA, shRNA, miRNA and proteins.[214] A study by Wahlgren et al. demonstrated the potential of plasma-derived exosomes for the delivery of siRNA to monocytes and lymphocytes.[215] Similarly, Andaloussi et al. reported that exosome-based delivery of siRNA can cross biological barriers.[216] Yim et al. reported that exosome engineering helps for efficient intracellular delivery of soluble proteins.[217] The study shows novel exosome as a protein carrier with high loading capacity and delivering efficiency.

Tumor cell-derived exosomes fuse preferentially with their parent cancer cells and can be used as Trojan horses for chemotherapeutic delivery.[218] The cellular uptake and target homing potential of exosomes are enhanced with the existence of vesicular surface proteins.[54,219] Exosomes have free access to agglomerate in leaky vasculature and solid tumor.[220] Moreover, the permeability of exosomes into a tumor cell is 10 times more than synthesized liposomes formulation.[219] Qiao et al. demonstrated cancer derived exosomes have potential to target their parent tumors and deliver drugs selectively to their parent cells.[218] The exosomal integrins involve in the adherence of exosome to the recipient cancer cell. Exosomal delivery of DOX enhanced the antitumor efficacy and also showed less off-target accumulation in the heart.[218] Toffoli et al. demonstrated that the exosomal delivery of DOX decreases the off-target cardiotoxicity compared with free DOX.[221] Ayuko et al. explained that exosomal integrins determine the organotropism effect of exosome into specific tissue. The fusion of specific exosomal integrins with cell-associated ECM mediates the exosome uptake into specific target.[222] A study by Tian et al. demonstrated the use of immature dendritic cell-derived exosome to target αV-integrin positive cancer cells, and the targeted exosomes showed high affinity both *in vivo* and *in vitro* experiments.[196] Tetraspanins, which are membrane glycoproteins on the surface of exosomes, can aid in recipient cell binding and fusion.[223] The minor differences of tetraspanin complexes can have a significant role for target isolation, fusion, selective cellular uptake and internalization through ligand-receptor interaction.[224] Tetraspanin 8 (encoded by Tspan8 gene) might have different internalization than CD9 and CD51.[225] Rana et al. demonstrated that CD11b and CD54-positive cells preferentially attach with Tspan8 expressing exosomes.[226] The tetraspanin web, a complex network of tetraspanin protein-protein interactions, is thought to contribute to the efficient and selective cellular uptake of exosomes. The cytotoxicity of DOX has also assessed in MDA MB-231and HCT-116 cell lines *in vitro* and an MDA MB 231 xenograft mouse model. The data revealed that the exosomal delivery of DOX reduces the systemic off-target cardiotoxicity. A report by Hirsh et al. showed that embryonic stem cell-derived exosomes increase the antiproliferative activity of DOX in breast cancer cell lines (MCF-7, MDA MB-231 and MDA MB-468).[227] The systemic administration of DOX showed enhanced anti-proliferative activity without systemic toxicity to normal

cells.[228] Kim et al. demonstrated that cancer derived exosomes may mimic the cancer membrane structure and initiate their internalization for effective treatment.[229]

Exosomes have aqueous core and lipid bilayers that help to encapsulate both hydrophilic and lipophilic cargo.[230–232] Exosomal encapsulation increases the drugs solubility, stability, and bioavailability.[233,234] Saari et al. demonstrated that exosomes loaded PTX showed higher cytotoxicity effect than exosome free PTX to autologous cancer cells.[235] PTX encapsulated in MSC derived exosomes showed an increased cytotoxic effect on human pancreatic adenocarcinoma cell lines.[236]

Interestingly, exosomes prefer acidic conditions for high cellular release, fusion and uptake.[237–239] Exosomes can help to overcome a pH-gradient-related challenges in chemotherapeutic drug delivery. Exosomes also have the potential to target specific cancer, deliver specific drugs and by-pass the drug efflux system.[240] A study by Kim et al. indicated the potential benefit of exosome to overcome MDR efflux pump effect of cancer cells.[240] This study demonstrated exosomal encapsulated PTX was useful to overcome Pgp-mediated drug efflux. The accumulation of exosomal DOX was higher than free DOX in MDR cells and also showed up to 30 times higher cellular internalization than liposome.[195] Alvarez-Erviti et al. reported the resistance of exosomes to RES and its ability to cross multiple biological barriers. The study also showed the delivery of siRNA to the mouse brain with dendritic cell-derived exosome through systemic injections.[197] Kalimuthu et al. showed the potential benefit of MSC-derived exosomes convey PTX to MDA-MB231 and showed enhanced effect than free PTX in nude mice model.[241] MSC-derived exosomes showed suppression of tumor growth and demonstrated successful targeting for colon cancer and human pancreatic adenocarcinoma.[242]

A. Exosomes for Lung Cancer

Exosomes have been studied as a new option for delivery of drugs for lung cancer.[243,244] Exosomes can avoid the issue related to toxicity, clearance, and bioavailability. Exosomes have been reported as potential delivery carriers for drugs like curcumin, PTX, celastrol, and anthocyanidins to lung cancer.[244] Aqil et al. reported that exosomal formulation enhances therapeutic response of celastrol against lung cancer cells. The exosome loaded celastrol, which is a plant-derived triterpenoid has shown significant anti-cancer effect than free drug. Furthermore, the *in vivo* data show that the systemic toxicological effect of exosomal delivery was also lower than free celastrol. The systemic toxicity was assessed by liver and kidney function test parameters.[245] Similarly, bovine-milk-derived exosomes were used to deliver curcumin to *in vitro* lung cancer cells and showed enhanced tissue distribution and efficacy with lesser systemic toxicity.[209] A study by Munagala et al. demonstrated the ability to load anthocyanidins (aglycones) into milk-derived exosomes. Anthocyanidins, which are extracted from plants, have antiproliferative, apoptotic, anti-inflammatory and antioxidant properties. Exosomes loaded with anthocyanidins showed significantly higher anti-proliferative effect with an ~ 4- to 60-fold decrease in IC_{50} compared with free drug.[246]

B. Exosomes for Breast Cancer

Globally, breast cancer is the most prevalent and second leading cause of mortality next to lung cancer. Mainly, it is the top cause of cancer-related deaths in the female population. Chemotherapy, hormonal therapy, radiotherapy and surgical resection are the existing treatment options currently available.[247] Chemotherapeutics like DOX, epirubicin, docetaxel (DTX), PTX, carboplatin, cyclophosphamide, and fluorouracil help to treat breast cancer.[248] However, drug resistance and toxicity are the most common challenges of the available treatment options.[249,250] Better treatment options and delivery methods are vastly needed for the treatment of breast cancer. Nanomedicine such as exosome-based drug delivery is a promising new therapeutic approach for breast cancer chemotherapy.[251] Gong et al. demonstrated the functional exosome-mediated co-delivery of DOX and thermophilically modified miRNA for triple-negative breast cancer therapy.[213]

The immature dendritic cell-derived exosomes showed the delivery potential of DOX with higher efficacy. Exosomes isolated from mouse and modified with RGD peptide have shown lower immunogenicity reported by Tian et al.[228] A similar study by Toffoli et al. showed that exosomal delivery of DOX had less cardiac toxicity than free drug.[252] MSC-derived exosomes were also tested for PTX delivery into MDA MB-231 cells, and the result was promising to use exosomes as chemotherapeutic drug carrier for better efficacy. Table 2 highlights the exosome-based drug delivery systems under clinical investigation.

IX. METHODS FOR ISOLATING EXOSOMES

Cellular debris, apoptotic bodies, exosomes, and other EVs are found together in the biological fluids. Exosomes with physical properties such as density, shape, size and surface proteins are the basis for isolation mechanisms.[37] Combining two or more isolation methods can help to separate exosomes from other interfering components efficiently.[253,254] Table 3 shows the physical properties of exosomes and other EVs such as micro-vesicles (MVs) and apoptotic bodies.

A. Ultracentrifugation

Centrifugation is a process which utilizes different centrifugal forces to sediment particulate constituents according to their density, size and shape. Ultracentrifugation is a centrifugation process which uses up to 6×10^6 *g* centrifugal forces and helps to isolate much smaller components such as; ribosomes, proteins, viruses, exosomes and other EVs. Acceleration (*g*), radius of rotation, a viscosity of the sample, and a sedimentation path length are the determining factors for efficient isolation of exosomes. Reports showed that particle size fraction between 20 and 250 nm can be isolated by ultracentrifugation and the subsequent centrifugation or size exclusion process yields the desired exosomes.[255,256] The ultracentrifugation method is considered as a gold standard method for exosome isolation and exosomes need 1×10^5 to 2×10^5 *g* centrifugation for 1 hour.[257,258] During the sequential centrifugation process, MVs, apoptotic bodies,

TABLE 2: Exosome–based drug delivery under clinical investigation

Study	Title	Disease	Trial ID	Phase	Year
1	A pilot clinical study on inhalation of mesenchymal stem cell exosomes treating severe novel coronavirus pneumonia	SARS CoV–2	NCT04276987	Phase I	2020
2	Allogenic mesenchymal stem cell derived exosome in patients with acute ischemic stroke	Acute ischemic stroke, cerebrovascular disorders	NCT03384433	Phase I/II	2017
3	Evaluation of adipose derived stem cells exosomes in treatment of periodontitis	Periodontitis	NCT04270006	Early Phase I	2020
4	Effect of plasma derived exosomes on cutaneous wound healing	Cutaneous ulcers	NCT02565264	Early Phase I	2015
5	Study investigating the ability of plant exosomes to deliver curcumin to normal and colon cancer tissue	Colon cancer	NCT01294072	Phase I	2011
6	Exosomes and Immunotherapy in non–Hodgkin B–cell lymphomas (ExoReBLy)	Lymphoma, B–cell, aggressive non–Hodgkin (B–NHL)	NCT03985696	NA	2019
7	Trial of a vaccination with tumor antigen–loaded dendritic cell–derived exosomes (CSET 1437)	Non–small–cell lung cancer	NCT01159288	Phase II	2010
8	Pilot immunotherapy trial for recurrent malignant gliomas	Malignant glioma of brain	NCT01550523	Phase I	2012
9	Isolation and characterization of the extracellular vesicles secreted by the human endometrium	Exosome collection	NCT02797834	NA	2016
10	MSC–Exosomes promote healing of MHs (MSCs)	Macular Holes	NCT03437759	Early Phase I	2018

TABLE 2: (*continued*)

11	Effect of micro–vesicles and exosomes therapy on β–cell mass in Type I diabetes mellitus	Type I diabetes mellitus	NCT02138331	Phase II/III	2014
12	A safety study of Intravenous stem cell–derived extracellular Vesicles (UNEX–42) in preterm neonates at high risk for BPD	Bronchopulmonary Dysplasia	NCT03857841	Phase I	2019
13	Exosomes in treating participants with metastatic pancreas cancer with KrasG12D mutation	Metastatic pancreatic adenocarcinoma, pancreatic ductal adenocarcinoma, stage intravenous pancreatic cancer AJCC v8	NCT03608631	Phase I	2018
14	Clinical trial of tumor cell–derived microparticles packaging chemotherapeutic drugs to treat malignant pleural effusion	Malignant pleural effusion	NCT02657460	Phase II	2016
15	Safety and effectiveness study of tumor cell–derived microparticles to treat malignant ascites and pleural effusion	Malignant pleural effusion, malignant ascites	NCT01854866	Phase II	2013

TABLE 3: Physical properties of exosomes, micro–vesicles (MVs), and apoptotic bodies

EVs and sizes	Biogenesis methods	Density/sucrose gradient	Centrifugal force used	Differential markers	Refs.
Exosome 30–100 nm	ESCRT dependent ESCRT independent	1.13–1.19 $^{-}$g/mL	1–2 × 10^5 g	CD81, CD63, CD9, Syntenin I, ADAM10, TSG101 EHD4, Annexin XI	261–263
Microvesicles 100–1000 nm	Cell surface blebbing	1.25–1.30 g	1 × 10^4–2 × 10^5 g	Actin–4, ARF6, VCAMP3	261,262, 264
Apoptotic bodies 1–5 µm	Apoptotic pathway	1.16–1.28 $^{-}$g/mL	1 × 10^4–2 × 10^5 g	S1PR1/3, Motilin, Phosphatidylserine, Actin–4, Annexin V, C3b, Thrombospondin	264,265

cell debris, cells, and other particles with higher buoyant density sediment before exosomes.[257] However, it is essential to note that due to overlapping densities and sizes, most current methods can only enrich for small EV (i.e., exosomes). Figure 4 shows a schematic diagram of the ultracentrifugation method for exosome isolation.

B. Density Gradient Ultracentrifugation (DGUC)

It helps to isolate and analyze nano-scale materials based on the size, structure and morphology differences. DGUC "refines" the isolated vesicles and uses a density gradient medium with a density of 1.07 g/mL or less.[258] Iodixanol in water, ice-cold PBS, and sucrose are the common gradient medium which are used for exosome isolation.[259,260] There are commercially available iodixanol density gradient fractionation membranes which are used to separate exosomes from non-vesicular components. The biological suspension is added to the gradient medium and components layered with complete gradient. DGUC helps to isolate samples with identical gradients. Apoptotic bodies, protein aggregates, and other non-exosome micro vesicles may interfere with the final isolated exosome product through ultracentrifugation.[259] DGUC helps to overcome the limitations of ultracentrifugation and gives the purest exosomes.[266,267] Kang et al. showed the application of DGUC for refined and high-performance exosome isolation method.[260] Briefly, after the isolation of cellular debris, applying 1×10^5 *g* for 3 hours with 60% iodixanol and then centrifuging with 1×10^5 *g* for 18 hours with 40% iodixanol helps to form up to 12 iodixanol gradient fractions and two exosome fractions. Ultracentrifugation uses a continuous density gradient or a stepwise gradient to minimize the interferences of those particulate materials from exosomes.[264]

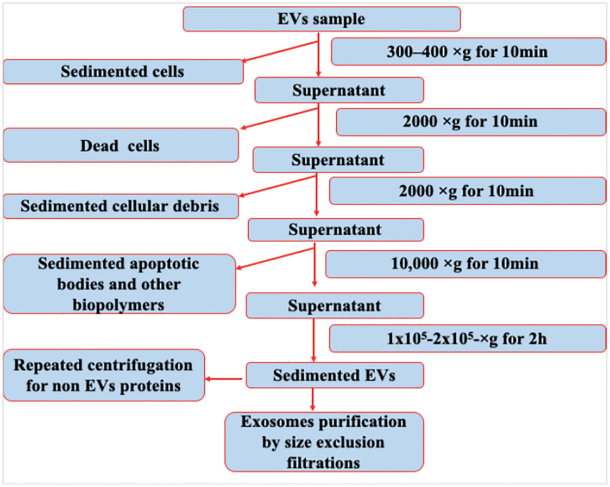

FIG. 4: Exosome extraction from biological fluids. Shown are ultracentrifugation methods to isolate exosome from cells.

C. Filtration

The filtration approach solely depends on molecular weight or sizes of components and helps to get optimum exosome yield.[268,269] Ultrafiltration, gel filtration and hydrostatic dialysis are included under filtration principles of exosome isolation. Exosomes can be isolated from the other EV components and cellular debris based on their defined molecular weight or size exclusion limit with a standard membrane filter. There are commercially available polyvinylidene carbonate membrane filter with various pore size ranges for osmosis, nanofiltration and microfiltration application.[270]

D. Ultrafiltration

It uses membrane filters with pore sizes of 50–450 nm to isolate exosomes from cellular debris and larger EVs.[271,272] By using nano-size filter membrane, targeted size exosomes can be isolated. Ultrafiltration method is commonly used as a subsequent step of ultracentrifugation and as a final step to gel filtration and chromatography.[273,274] Ultrafiltration can be processed either by tangential flow filtration (TFF) or by direct flow filtration (DFF) methods.[275] The DFF method, which is also called dead-end filtration, has membrane fouling and impaired particle separation problem. Moreover, DFF is preferred only for small volume samples, for example, up to 30 mL.[276]

TFF, which also called as crossflow filtration, is a more rapid, efficient and convenient process to isolate large scale exosomes volumes.[276,277] TFF is the process in which the sample fluid flows tangentially across the filter membrane and avoid cake or clog formation. Briefly, samples are filtered through a 0.2-µm polyethersulfone (PES) membrane, larger particulate matter like cell debris and apoptosis bodies are detained out. Then, the semi-processed filtrate sample including exosomes is subjected to the ultrafiltration process through a TFF system by 500-kDa molecular weight cutoff cartridge with 120 mL/min feed-flow rate, < 3.5 psi transmembrane pressure, and > 10:1 crossflow rate.[276] The exosomes are too large to pass through the pores and kept as retentate. Conversely, small molecules, including free proteins, pass through hollow fiber pores and are eluted as permeate and ultimately discarded from the process.[278] For quality exosome isolation and purification, the retentate samples are re-concentrated serially by TFF and contaminants smaller than the 500 kDa are depleted.[278] Finally, the purified exosomes are resuspended and stored in 0.1M sucrose in a polyethylene terephthalate glycol (PETG) bottle at –80°C and used for the desired analysis. Generally, exosomes can be isolated successfully by combining different isolation methods such as ultrafiltration with ultracentrifugation or centrifugation with ultrafiltration.[253] Filtration with lower pore diameter membrane and ultracentrifugation provides pure exosomes.[279]

E. Hydrostatic Filtration Dialysis (HFD)

It helps mainly to isolate the whole EVs from highly diluted solution without ultracentrifugation process.[280] Musante et al. showed HFD protocol for isolation of EVs from urine

samples.[281] At the initial step of HFD, samples are centrifuged with 2000 × *g* to remove cells, bacteria, and debris as a pellet. Then, the supernatant is kept in dialysis membrane (1000 kPa) and particles of 1000 kPa or less pass through the membrane accordingly with hydrostatic pressure differences. Finally, exosomes vesicles are sedimented with 40,000 × *g* by centrifugation. In HFD isolation process, exosome fractions recover in an early stage and it is considered as an advantage compared with multistep centrifugation. HFD is also considered as an efficient method compared with ultracentrifugation.[282,283]

F. Size-Exclusion Chromatography (SEC)

This method mainly helps to remove protein and lipoprotein impurities from isolated exosomes.[284,285] It has been used to separate exosomes from urine and blood plasma proteins. It is used as a subsequent isolation method to ultracentrifugation and ultrafiltration. Sepharose 2B/CL4B, qEV and Sephacryl S-400 are the columns which are generally used to isolate exosomes by gel filtration chromatography.[264] SEC can be done under low pressure, which helps to isolate exosomes with intact integrity.

G. Precipitation

It is a method which is based on charge precipitation of exosomes from biological fluids. Negatively charged exosomes can interact with positively charged protamine in PEG 35,000 Da matrix and form precipitate.[286] The recovery and resuspension of exosomes are more efficient than ultracentrifugation-based isolation.[287] Kanchi et al. reported the potential benefit of polymeric precipitation method to isolate urinary exosomes with less labor and without expensive equipment.[288] This method first utilizes DL-dithiothreitol solution to reduce or remove the polymeric networks of Tamm-Horsfall protein and then precipitation of exosomes can be achieved with only 10,000 × *g* centripetal force at 25°C and 30 min. There are also commercial kits available which utilize polymeric precipitation approach and eliminate ultracentrifugation. ExoQuick™, Exo-spin™ are the commercially available total exosome isolation reagent from Invitrogen™ and miRCURY™.[289]

H. Immunoaffinity Interactions

Immunoaffinity method is an ideal method to isolate pure exosome with the principle of immunoaffinity interaction between the exosome surface proteins (antigens) and their target antibody or isolating ligands molecules.[290] It helps to isolate a specific type of exosomes according to their surface markers. Microplate-based enzyme linked immunosorbent assay (ELISA) is an example of immunoaffinity isolation kit which is used to isolate exosomes according to the exosome surface markers. Tetraspanin proteins are the determining factors for this isolation method. Anti-CD9, anti-CD63, and anti-CD81 are common examples of antibodies used in immunoaffinity exosome isolation. Tauro et al. showed that immunoaffinity method can be used to isolate exosomes from colon cancer

cells with higher efficiency than ultracentrifugation and density gradient isolation.[291] Zarovni et al. reported an exosome isolation method with an antibody-coated, magnetic particle-based technique for isolation of exosomes.[292]

I. Microfluidic-Based Isolation

Microfluidic based exosome isolation may include immune-affinity approaches for exosome trapping, nano-porous membrane sieving approaches or nanowire on micropillar for exosome trapping. All three systems require chips with viscoelastic analysis and electric manipulation for the exosome isolation process. The specificity of exosomes is high, with microfluidic chip-based immunoaffinity capturing methods. Exosomes with size range between 40–100 nm are specifically entrapped with this microfluidic chip. Kanwar et al. reported the benefit of ExoChip for exosome isolation and quantification.[293] Sieving approaches can be used to filter exosomes from the whole blood with pressure or via electrophoresis.[294] The utilization of pressure makes separation time shorter, and the electric field results in high exosome purity. Specificity, reproducibility, low isolation time, and less isolation cost are some of the benefits of microfluidic-based exosome isolation.[295]

X. EXOSOMAL DRUG-LOADING METHODOLOGIES

Generally, drugs can be sorted into exosomes by two methods: active loading and passive loading.[46,125] Figure 5 shows the schematic diagram of the pre-drug loading and post-drug loading methods. Active loading method is also termed as remote or post-loading

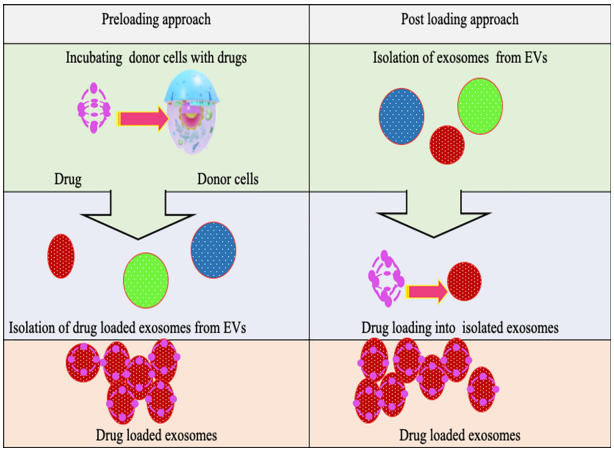

FIG. 5: Exosomal drug–loading approaches. Drug–loaded exosomes can be secreted in pretreated cells or tissues, and exosomes with encapsulated drugs can be isolated from cell secreted EVS. Drugs can also be loaded in to ready–made or isolated exosome by active/passive drug–loading methods.

approaches.[232] It is a method in which the drug loading process is performed with isolated exosomes. The passive loading approach, which is also called preloading method, deals with the secretion of drug-loaded exosomes from a pretreated donor or source cells.[232] The passive loading method does not require the addition of active substances into the system. The active loading approach has been proven to be more effective in achieving a higher drug-vesicle ratio due to its active pumping mechanisms.[193] Conversely, the post loading approach is more suitable for hydrophobic drugs than hydrophilic drugs.

A. Passive Loading Approaches

1. Incubation of Drug with Targeted Exosome Donor Cells

Target cells are treated with a desired drug and then drug loaded exosomes are secreted from the pretreated cells.[93,296] Pascucci et al. demonstrated the secretion of PTX loaded exosomes from pretreated SR4987 mesenchymal stroma cells.[297] In the study, first the cells were treated and incubated with low dose PTX for 24 hours, and then the cells were washed and reseeded in new flask with fresh medium. Finally, the PTX-loaded exosomes were isolated and collected from the cell culture medium after 48 hours. For release of drug loaded exosomes, the pretreated donor cells may be exposed to biological or mechanical stimuli such as heat, hypoxia and ultraviolet light or combination.[298–301]

2. Incubation of Drug with Isolated Exosomes

This approach is referred as a passive method of loading. Drugs and exosomes are simply incubated together for some time and the drugs diffuse into the exosomes due to concentration gradient.[125] This method is suitable for loading hydrophobic drugs and the drug loading efficiency is directly related with hydrophobicity of drugs. Dongmel et al. showed the curcumin loaded exosome by incubation method.[190] Exosomes and curcumin were mixed in PBS and incubated for 5 min at 22°C and the mixture was centrifuged based on different sucrose gradient. The solubility, stability and bioavailability of curcumin were enhanced by exosome encapsulations as compared with free curcumin.[190] Similarly, enzyme catalase was loaded into exosomes by incubating in PBS buffer for 18 hours at room temperature.[200]

B. Active Drug-Loading Approaches

1. Incubation with Membrane Permeabilizers

This method uses membrane permeabilizer surfactant like saponins.[296,302] Briefly, the interaction of cholesterol and a surfactant leads to exosomal membrane permeability.[93,193] It is suitable to load small hydrophilic molecules into exosomes up to 11-fold higher than passive incubation method. Compared with simple incubation, membrane permeability methods have enhanced loading efficiency.

2. Sonication

The isolated exosomes are mixed with drugs and then sonicated by using a homogenizer probe.[303] Drug loading by sonication method uses mechanical shear force to diffuse into the exosomes through deformed membrane.[125] Kim et al. reported that exosome membrane integrity decreases after sonication. Even though membrane microviscosity significantly decreased after sonication, the sonication process does not affect the membrane-bound proteins and lipid contents of the exosome.[240] Moreover, the integrity of the exosome membrane can be restored with 1-hour incubation at 37°C.

3. Extrusion

It is a type of post-loading approach which utilizes a syringe-based lipid extruder and has harsh mechanical force.[232,304] Briefly, the target drug and isolated exosomes are loaded into the extruder with 100- to 40-nm porous membrane at a regulated temperature, and then the drug mixed with disrupted exosome membrane. Fuhrmann et al. showed the benefit of extrusion type drug loading for exosomes.[305] Porphyrin was loaded into triple-negative breast cancer cells (MDA MB-231)–derived exosomes. The cytotoxicity effect of porphyrin was higher when loaded with extrusion type loading compared with incubation method. Result demonstrated that extrusion method loading modified the zeta potential of the original exosomes. The number of extrusion processes have contribution for effective drug loading.

4. Freeze and Thaw Cycles

This method has three steps: incubation of isolated exosome with drug for a given time at room temperature, freeze at –80°C or below (liquid nitrogen), and finally thaw at room temperature.[188,232] For better drug loading, the mentioned process is repeated at least for three cycles. Compared with sonication or extrusion methods, the freeze/thaw loading method has lower drug loading capacity.[125]

5. Electroporation

This method utilizes an electrical field which disturbs the phospholipids bilayer of exosomes and creates small pores on it.[207,306] The most efficient electroporation protocols use 750 V and10 pulses.[307] The drugs can then be diffused or sorted through the developed pores, and meanwhile, the membrane is recovered after loading. Larger molecules such as nucleotides (siRNA/miRNA) can be loaded into exosomes by electroporation loading approaches.[215,305] Electroporation also helps to load smaller hydrophilic molecules. It has low loading capacity due to the issue related with RNA aggregation and exosome instability.[305]

XI. CHARACTERIZATION OF EXOSOMES

A. Transmission Electron Microscopy (TEM)

The principle involved in TEM is similar to that of the light microscope. However, TEM uses electrons with smaller-wavelength than that of light. The optimal resolution is far better than that of light microscopy.[308] TEM is known to be the gold standard to study the morphology of exosomes by electron microscopic imaging, due to small size of exosomes and ease of sample preparation. TEM has a resolution of 1nm with broad beam to focus the sample, negative staining is considered to be simple and rapid, lasting for 2–3 hours to generate two-dimensional image. Briefly, exosomes are first fixed in 2% w/v paraformaldehyde, deposited on carbon coated Formvar grids, and incubated for 20 min. The precoated grids are then washed with phosphate buffered saline and incubated with crosslinking agent like glutaraldehyde and washed with water. The exosome vesicles are then stained with 2% w/v of uranyl acetate solution and air dried.[309] TEM can be used to: confirm the existence of exosomes in the solution, study the morphology of vesicles and to assess the quality of exosomes.[310] Because TEM has limitations such as lack of reproducibility and inefficiency, it is rarely used to quantify exosomes. Exosome surface markers can be identified and quantified by using immuno-electron microscopy,[311] it encompasses incubating exosome vesicles with primary antibodies and secondary antibodies attached to 5- to 40-nm gold particles. Cup-shaped morphology was reported for exosomes by TEM.[312]

B. Scanning Electron Microscopy (SEM)

The basic principle involved is an accelerated electron in an SEM carry significant amounts of kinetic energy, and this energy is dissipated as a variety of signals produced by electron-sample interactions when the incident electrons are decelerated in the solid sample. Sample scanning in SEM is line by line using fine point beam.[313] Here, SEM focuses surface of samples to get three-dimensional image of exosomes. Briefly, exosomes are fixed on carbon coated/copper grids with glutaraldehyde and dehydrated with ethanol. The grids are air dried and coated with gold using sputtering machine at a thickness range of 2 to 10 nm. Then the samples are ready to analyze by SEM.[314,315] Reports revealed that SEM imaging of exosomes is round and bulging in appearance.[316]

C. Cryogenic Electron Microscopy (Cryo-EM)

Cryo-EM is a type of TEM in which a typical sample preparation involves cryogenic temperature for native aqueous environment observation. For cryo-EM analysis, the liquid suspended exosomes are placed on a grid, then it is rapidly immersed in liquid ethane to allow the vitrification of exosome sample. After vitrification process, the samples can be directly analyzed for imaging by cryo-EM or transferred in liquid nitrogen for storage for further analysis.[317] Morphology of exosomes by cryo-EM was found to be bilayer structures and sometimes surrounded by smaller vesicles.[318,319]

D. Atomic Force Microscopy (AFM)

AFM uses a cantilever with a very sharp tip to scan over a sample surface. As the tip approaches the surface, the close-range, attractive force between the surface and the tip cause the cantilever to deflect towards the surface. AFM is useful for checking the topography of exosomes with high resolution less than 1 nm.[320] Briefly, an exosome vesicle suspension is placed on a mica substrate and allowed to air dry at room temperature. The dried samples are then washed with ultrapure water and allowed to dry in liquid nitrogen gas. Here the silicon probes are used to view the sample under AFM and analyzed with software.[321] This technique is useful to identify the specific receptor sites on exosome surface, for example, Sharma et al. reported multiple CD63 receptor sites on exosome using immunogold imaging by AFM.[322]

E. Nanoparticle-Tracking Analysis (NTA)

NTA is one of the most sophisticated methods for measurement of size distribution and concentration of sample by capturing the Brownian motion of particles in a video. Based on the different diffusion movements of large and small particles in the surrounding liquid, the hydrodynamic diameter of the particles is determined. NTA provides better and reproducible results compared with TEM and flow cytometry. NTA is able to detect the vesicles with diameter range of 30–1000 nm with high resolution. The basic principle involved in this is dynamic light scattering principle along with usage of Stokes-Einstein equation to quantify particle concentration and size.[323] As per the NanoSight NS300 equipped with a 488-nm laser (Malvern Panalytical, Malvern, UK) user manual, under the scattered settings, each sample is loaded by syringe pump with speed of 15 into the machine, Camera level was set at 11 and an analysis detection threshold of 3. Each video had a duration of 40 sec with frame rates of 25 frames/sec. The entire process of sample analysis consumes about 10–15 min to obtain the data of particle size distribution and concentration. NTA version 3.2 software was used to record and analyze the sample videos. All these analyzes are carried out quickly and statistically reliably as required in the scattered light or fluorescence mode.[324]

Electrophoretic light scattering principle (the movement of the particles in an applied electric field) was applied to measure the zeta potential of exosomes to evaluate the vesicle stability in solution state. ZetaView® set up will be used in NTA for zeta potential measurement. For NTA and zeta potential analysis samples will be diluted at 1:1000 in PBS, and vortexed/sonicated for 10 min at room temperature.[325]

F. Asymmetric Flow Field-Flow Fractionation Technology (AF4)

AF4 is the technique used to separate distinct subsets of extracellular vesicles such as exosomes based on their density and hydrodynamic properties. In this technique, exosomes are forced through a forward laminar channel and, based on their Brownian

motion sorted into different populations. Larger particles have lower diffusion rates and tend to move more slowly. However, smaller particles have higher diffusion rates and tend to move faster. Several studies have been reported for comparison between AF4 and NTA for exosome analysis.[326] AF4 was able to distinguish various vesicle subpopulations. including the discovery of a novel particles ~ 30 nm in size termed exomeres. But NTA, has shown a single, broad peak from 50 to 150 nm. Therefore, AF4 is a superior, advanced technique to address the size heterogeneity of exosome characterization.[327]

G. Resistive Pulse Sensing (RPS)

RPS is the technique that measures the size of individual vesicles in the fluid passed through small orifice based on their electrical resistance.[328] RPS is able to detect the vesicle size range of 50–1000 nm. Spectradyne, LLC is a leading company in the microfluidic measurement of colloidal particles. RPS has higher size resolution with better accuracy in measuring particle size distribution compared with dynamic light scattering and NTA techniques. The results obtained for same sample by RPS analysis are in close correlation with count obtained by TEM. Grabarek et al. showed that NTA failed to distinguish exosomes from liposomes, protein aggregates and bacteria and the concentrations are 5- to 10-fold higher compared with RPS analysis.[329]

H. Flow Cytometry

Flow cytometry is not a reliable technique to detect exosomes because of low resolution. Several studies have been reported that extracellular vesicle of size range of 300–500 nm can be semi-quantitatively detected based on their surface markers.[330] Briefly, exosomes were incubated for 15 min with aldehyde/sulfate-latex beads with continuous rotation for reaction. The reaction was stopped by adding glycine and bovine serum albumin (BSA) solution. Exosomes bound beads were washed with PBS and blocked with BSA solution. For a specific membrane marker detection primary antibodies and fluorescence labeled secondary antibodies were added in sequence. Incubation of exosome bound beads with an isotype control followed by a secondary antibody or in the absence of primary antibodies act as negative control. Using this method, Melo et al. reported that exosomes derived from non-tumorigenic cells carry less glypican-1 compared with exosomes derived from pancreatic cancer cells.[331]

I. Exo View Platform

Exo View is an antibody-based exosome array by Nano View Biosciences. This technique enables the differentiation of exosomes subpopulation with a very low sample

volume and is less time consuming compared with flow cytometric technique. Exosome sample or biological fluids containing exosomes are incubated with the chips overnight. After incubation, chips are rinsed with PBS on a shaker and air dried. Captured exosomes are identified using single particle interferometric reflectance imaging sensor technology. Characterization concepts using purified exosomes from a HEK 293 cell culture and clinical relevance of exosome characterization was demonstrated directly from human cerebrospinal fluids.[332] This technique allows enhanced contrast in the signal from particles. Antibodies against exosome surface markers are arrayed on silicon chips by this method.

J. Other Methods

In vivo tracking of exosomes can be done by using several methods such as labeling the exosomes with lipophilic carbocyanine dyes, including PKH67 and PKH26 (red) and the other method include labeling of exosomal markers like CD63 with green fluorescence protein (GFP) or mCherry in the cells.[333] The fate of fluorescently labeled exosomes in blood circulation of mice were detected using the IVIS Spectrum system.[334]

XII. CHALLENGES AND FUTURE DIRECTIONS

The research and clinical studies related to the utilization of exosomes for therapeutic drug delivery is still in its infancy, a more advanced understanding and systematic characterization methods are on demand. The significant limitations of exosomes as drug delivery agent is rotated on lack of standard extraction and loading methods, risk of delivering unwanted exosomes related cargo substances. Lack of standard exosome isolation protocol is leading to low purity and batch-to batch variations. Large scale exosome isolation is still challenging and it is a costly process to perform clinical studies.[258] Similarly, lack of standard drug loading protocol and low loading efficiency are the main challenges of exosome as cargo vehicle. There is not adequate proteomic data and understanding about exosome nature in the pathophysiological conditions. The study shows that, the unwanted biological effect of exosomes may promote drug resistance between cancer cells through horizontal transfer of miRNA.[335] Exosomes require clinical-grade standards and characterization and purification methods to ensure quantitative and qualitative exosome extraction and for the safe drug delivery process.

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