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REVIEW ARTICLE

Understanding exosomes: Part 1—Characterization, quantification and isolation techniques

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1 | INTRODUCTION

The smallest extracellular vesicles, exosomes, range in size from 30 to 150 nm and are found in the majority of bodily fluids.[1] They transport signaling molecules that drive a number of biological processes, including cell signaling, immunological responses, tumor metastasis, and other cellular activities. Exosomes have now been shown in several investigations to play both diagnostic and therapeutic functions, in which their precise detection, separation, and quantification are crucial. Today, exosomes are one of the most highly researched topics in regenerative medicine with over 5000 publications being published on the topic yearly (Figure 1). This comprehensive review aims to discuss their biogenesis as well as their standard isolation techniques, including ultracentrifugation, microfluidic, immunoaffinity, precipitation, size-exclusion chromatography, ultrafiltration technologies. The fundamentals of each isolation technique are detailed in length, along with their respective benefits and drawbacks. Furthermore, the quantification of exosomes by microfluidic devices, dynamic light scattering, electron microscopy, adjustable resistive pulse sensing, and flow cytometry are explained.

1.1 | What are exosomes?

Exosomes are intriguing extracellular vesicles in industry and biomedical research as possible disease biomarkers and therapeutic agents since they share components with their parent cells. Exosomes reflect the biophysical features of mesenchymal stem cells (MSCs), yet many therapeutic studies have demonstrated that they are considered more effective than MSCs themselves for a number of reasons. Notably, exosomes bypass important immune responses and are considered exponentially safer than MSCs will ever be. Their use has therefore been investigated in many university-based clinics along with private clinical practice despite lacking FDA and CE clearance. Crucial to their success in clinical practice remains in their ability to be manufactured under standardized Good Manufacturing Practices (GMP). Sensing technologies are now being used to characterize exosomes from the central nervous system (CNS), which have the ability to penetrate the blood–brain barrier.[2] These exosomes are being used in the early diagnosis of neurodegenerative illnesses and as a means to track disease development.[3]

Over 50 years ago, "platelet dust" was the term used to characterize extracellular vesicles (EVs).[4] The field more specific to the smallest EVs, exosomes, was further termed in 1983, with two research groups having been given credit for their discovery. In these studies, labeled transferrin receptors (TfRs) were followed as they moved from the plasma membrane into developing reticulocytes. Transferrin receptors are internalized and repackaged into very small (50 nm) vesicles once they reach their destination cells.[5,6] In contrast to previous assumptions, the authors found that vesicles were secreted out of maturing blood reticulocytes into the extracellular space, where they were later dubbed exosomes due to their vesicular exit from the cell.[7,8]

1.2 | Historical findings of exosomes

In the early 1980s, Trams et al. described exosomes as nanosized vesicles released during reticulocyte maturation.[9] The presence of transferrin bound to the exosome surface was later discovered and verified by Johnstone et al.[10] As a result, the concept of exosomes as a kind of excretory signal used by many cell types to

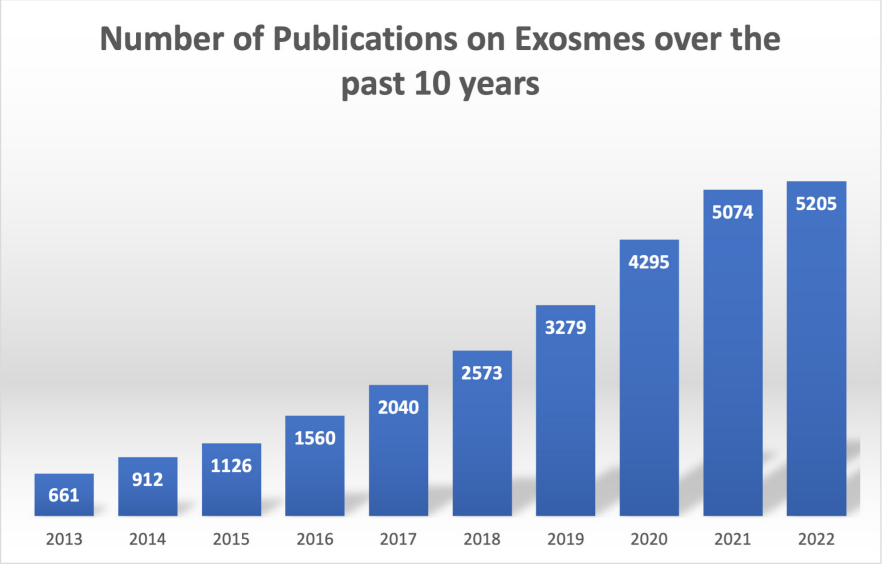

FIGURE 1 Number of publications on exosomes over the past 10 years (data from PubMed).

load RNA, proteins, lipids, etc., for intercellular transit gained traction.[11] In terms of their structure, EVs are characterized by a lipid bilayer that encloses a minimal amount of cytoplasm. The aqueous core of the lipid bilayer plays a role in the transfer of cargo.[12,13] In more recent years, the complexity of exosomal functions has been explored, and these vesicles have been found to have various roles in disease progression, immune responses, cell functions, and tissue regeneration.[14]

2 | BIOGENESIS OF EXOSOMES

Eukaryotic cells generate exosomes in their endosomes.[15] It is challenging to identify exosomes in a manner that separates them from other EVs, such as microvesicles and apoptotic bodies, because of the variety among exosomes and their overlap in features with different EVs.[16] EVs are biological nanoparticles encased in lipids that are secreted by almost every living cell. The role of EVs in many pathological and physiological intercellular signaling processes was not previously recognized but is now universally accepted as essential.[17,18] It is now generally accepted that EVs provide a physiologically relevant setting for the targeted delivery of a variety of biological substances to distinct cellular and tissue niches. Because of this, they have drawn much attention as having the potential for use in next-generation drug delivery methods.

The endosomal system is the site of exosome production. After developing into late endosomes or multivesicular bodies (MVBs), the endosomal membrane gives rise to intraluminal vesicles (ILVs; sometimes called exosomes) in the organelle's lumen (Figure 2).[19] The release of exosomes into the extracellular environment occurs during MVB fusion with the plasma membrane.[20] EVs come in three main sizes: exosomes (30–150 nm), microvesicles (MVs) or ectosomes (50–1000 nm), and apoptotic bodies (500-2000 nm) (Table 1). Exosomes are secreted with the formation of endocytic vesicles, which bud inward. These sacs will consist of extracellular fluid inside and a cell membrane outside.[5] In recent years, exosomes have garnered much interest in drug delivery since they can be utilized to target specific organs based on their surface receptors, which has led to considerable improvements in therapeutic medicine.[21,22] Typically, exosomes have important proteomic and genetic information that plays a key role in transporting cargo toward the targeted tissue/organ. Based on these discoveries, exosomes have been isolated and further reused for therapeutic medical applications with great success. Exosomes reach target tissues via three primary means. Exosomes activate target cells by attaching to particular ligands present on their receptors, a process known as L-R binding (L-ligand, R-receptor). Using the budding process, exosomes may also transfer cell surface receptors to the recipient cell. Finally, membrane fusion permits the horizontal transfer of the donor cell's cytosolic contents to the recipient cell.[23]

Therefore, a thorough understanding of exosome biogenesis pathways is important for the development of associated treatment approaches. Activation of a growth factor receptor on the cell membrane is the first step in the exocytosis process by which cells actively produce exosomes. After being stimulated, this receptor activates its target protein, and the ligand–receptor complex is internalized by endocytosis. There are typically three phases involved in the production of exosomes.

1. The endocytic cell wall contributes to the production of endocytic vesicles.

2. The formation of an early endosome involves inward budding of the membrane of an endosomal vesicle, which results in the formation of multivesicular bodies (MVBs) and proteins such as clathrin.[24,25] Intraluminal vesicles (ILVs) are larger vesicles that, after maturation, transform into late exosomes.

3. Exosomes are the vesicular contents discharged into the extracellular environment when MVBs fuse with the cell membrane.[26,27] The production of exosomes by vesiculation is a relatively uncharted area of study. Released exosomes are taken up by cells in a targeted manner determined by the proteins they express on their surface.[28]

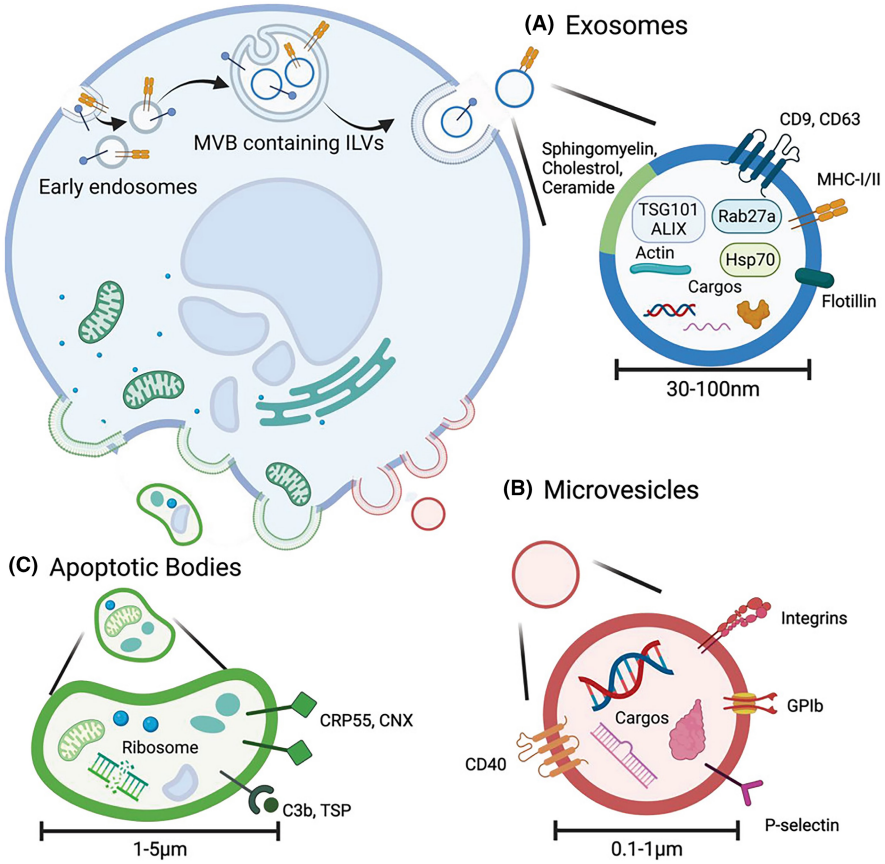

FIGURE 2 Biogenesis pathways and biochemical compositions of (A) exosomes, (B) microvesicles, and (C) apoptotic bodies. (A) Proteins, lipids, and genetic material are loaded into ILVs, which are eventually released from the parent cell as exosomes. (B) Microvesicles are formed by direct budding off of the plasma membrane and contain proteins, lipids, and genetic materials. (C) Apoptotic bodies bud directly from the plasma membrane during apoptosis and consequently contain higher amounts of disintegrated organelle content. Reprinted with permission from Lai et al.[337]

TABLE 1 Comparison between extracellular vesicles.

Types	Apoptotic bodies	Microvesicles	Exosomes
Origin	Plasma membrane	Plasma membrane	Endocytic pathway
Size[a]	500–2000 nm	50–1000 nm	30–150 nm
Function	Facilitate phagocytosis	Intercellular communication	Intercellular communication
Markers	Annexin V, phosphatidylserine	Integrins, selectins, CD40	Alix, Tsg101, tetraspanins (CD81, CD63, CD9), flotillin
Contents	Nuclear fractions, cell organelles	Proteins and nucleic acids (mRNA, miRNA, and other noncoding RNAs)	Proteins and nucleic acids (mRNA, miRNA, and other noncoding RNAs)

[a] Extracellular vesicles are typically defined based on their size.

In particular, the presence of tetraspanin–integrin protein complexes on the exosomal surface adds to the selectivity by which exosomes target cells.[29] Increased expression of proinflammatory receptors on cell surfaces promotes the selective absorption of exosomes by target cells.[30]

3 | STRUCTURE AND COMPOSITION OF EXOSOMES

Exosomes are so small that they can only be seen under an electron microscope, making them invisible to the human eye and light microscopes. Because of the extensive dehydration procedures required for electron microscopy processing, the exosomes appear as flattened spheres.[31,32] Recent structural investigations of exosomes have shown the presence of certain lipids that keep them biologically active.

The macromolecular components of exosomes include proteins, lipids, messenger RNAs (mRNAs), and microRNAs. These exosomes are created by their parent cells,[26] and investigators have now identified over 8000 proteins and 194 lipids that have been found within exosomes.[33]

Exosomes released from cells including immature dendritic cells, intestinal epithelial cells, B lymphocytes, and others, contain both ubiquitous and cell-specific proteins.[34] Rab proteins (Ras superfamily of monomeric G proteins) and other ubiquitous proteins found on exosomes aid in their fusion with the membranes of other cells and binding with target proteins.[35] Exosomes also include a variety

of other proteins, including annexins I, II, V, and VI, which all play important roles in cell dynamics and membrane fusion.[36,37] Cytoskeletal proteins, GTPases (albumin, moesin, synenin, and actin), tetraspanins (CD82, CD8, CD63, and CD9), heat shock proteins (Hsc90 and Hsc73), apoptosis proteins, and adhesion molecules[38] are also found in exosomes from different sources.[39] Integrins are found on the surface of exosomes and play a crucial role in exosome fusion with particular target cells.[40] Furthermore, these proteins serve as the information payload when exosomes are shuttled between cells.

In addition to proteins, exosomes contain an abundance of lipids. Exosomes consist of a lipid bilayer composed of sphingolipids (ceramide and sphingomyelin),[41] phospholipids (phosphatidylserine, phosphatidyl ethanolamine, and phosphatidyl choline),[42] phosphatidylinositol and mono-sialo-tetra-hexosylganglioside (GM3).[43] In comparison to the original cell from which they were isolated, exosomes have a lipid content that is approximately four times higher. Phosphatidylserine, in the form of different phospholipid transporter enzymes, is expressed on the plasma membrane of exosomes, whereas sphingomyelin and GM3 are primarily responsible for exosome membrane stiffness.[44,45] Lipids of this kind also play a role in the binding of the outer proteins, which is necessary for exosomes to send signals and fuse with the plasma membrane.[46] Exosomes include bioactive lipids, such as prostaglandin and leukotriene, as well as activated enzymes involved in lipid metabolism.[47] Nucleotides are also found in noncoding RNAs, microRNAs (miRNAs), and messenger RNAs (mRNAs).[33] The strength and stiffness of the exosome membrane, which make it less vulnerable to breakdown outside the cell and more durable as a carrier, may be due to the high quantities of sphingomyelins and unsaturated lipids in the membrane.[48] Thus, exosomes offer better stability in the human body than the signaling molecules currently used for delivery, such as recombinant human proteins. Many studies have investigated the 8000+ different proteins and lipids contained within exosomes, including their various ratios, and have been identifying how various combinations can be utilized for therapeutic breakthroughs.

3.1 | Molecular composition of exosomes

Modulation of the protein cargo in released exosomes may be affected by factors such as the microenvironment's mechanical characteristics, biochemical impulses, and topography, in addition to the cell type of origin.[49] Media composition, mechanical stress, disease type, and oxygen levels are just a few of the environmental elements that might affect exosome secretion and composition.[50]

3.2 | Protein markers in exosomes

Typically, there are fewer than a hundred proteins in a single EV.[33] The number of EVs needed to produce 1 μg of total protein is approximately ≈10^9–10^{10}.[51,52] EVs of the same type have widely varying protein cargos,[53] but some proteins are ubiquitous in these EVs owing to their functions in EV synthesis and protein sorting.[54] Thus, proteins can serve as indicators in clinical diagnostics and EV characterization, allowing for the differentiation of apoptotic bodies, microvesicles, and exosomes.[55] Common tetraspanin proteins on exosome membranes include CD8, CD63, and CD9; these proteins are involved in protein trafficking, signaling, and membrane fusion.[56,57] Exosome biogenesis is a multistep process that also involves TSG101, flotillin, and ALIX.[58] The ESCRT protein complex and related proteins Hrs, flotillin, TSG101, and ALIX are all found in exosomes and play important roles in MVB formation and ILV engulfment. The binding of MVBs to the plasma membrane and the modulation of exosome secretion are mediated by the vesicle fusion and transport proteins annexin and Rab27a.[59,60] Exosomes also contain abundant heat shock proteins (Hsp70 and Hsp90) and cytoskeletal proteins (actin and myosin).[22,61] These proteins may be utilized to identify and isolate exosomes because they act as markers that distinguish them from other extracellular vesicles.

3.3 | DNA and RNA in exosomes

The nucleic acids in EVs are transcribed and translated and ultimately affect the behavior of the cells to which they are delivered.[58,62] The restoration of metabolic function in breast cancer cells that had been reduced by hormone treatment may be attributable to mitochondrial DNA transported by exosomes.[63] EVs have a role in both healthy and diseased cell–cell interactions, and they often carry miRNA and mRNA.[13] Viruses may be found in the RNA of exosomes released from infected cells, and the miRNA profiles in exosomes released from malignant cancer cells differ from those of normal cells.[64] The potential of using these RNAs as early illness indicators is the subject of intensive research for the early diagnosis of cancer.

Since EV subtypes vary in their nucleic acid composition,[65] it is challenging to create a genetic profile for each EV subtype. Nevertheless, there are a few consistent differences in nucleic acid composition among EV subtypes. Exosomes and microvesicles contain an abundance of miRNA and mRNA,[66] whereas apoptotic bodies include an abundance of ribosomal RNA (rRNA).[67] Complete genomes can be found in microvesicles and exosomes[68,69]; however, in apoptotic bodies, DNA is in pieces.[70] Up to ≈500 unique miRNA molecules have been found in a single exosome.[71] The composition and genetic profiles of exosome nucleic acids, as well as how they vary from other EVs, are still not well understood.[72,73] To enhance and standardize the separation of exosomes from other EVs, it is necessary to conduct a large-scale comparison of EVs to find variations in nucleic acid content.

3.4 | Lipids in exosomes

Unlike their protein and nucleic acid contents, the lipid composition of EVs is not as well known. Mass spectrometry has uncovered hundreds of different lipid molecules in EV membranes.[74] In

platelet-derived exosomes, the most common lipids are sphingomyelin (12.5%), phosphatidylcholine (15.9%), and cholesterol (42.5% of lipids) and their derivatives.[42,75] The lipid compositions of exosomes diverge significantly from those of their parent cells,[42,76] yet only marginally so among exosomes from the same cell line.[42] Exosomes are efficient transporters of proteins and nucleic acids because their plasma membranes are more solid and resistant to degradation than their parent cells. This may be due to the increased amounts of cholesterol, desaturated lipids, and sphingomyelin in exosome plasma membranes.[77] There are different amounts of lipids on both the outer and inner leaflets of the exosome membrane. This helps the exosome remain stable.[78] Microvesicles, in contrast to exosomes, contain lipids that are identical to those of their parent cells. Additionally, apoptotic bodies contain more phosphatidylserine than their parent cells.[79]

Lipids in EVs have a regulatory role as well.[66] The "eat me" signal sent out by phosphatidylserine in the outer membrane of apoptotic entities is a well-known example.[80,81] Another way lipids may affect inflammation is via their anti-inflammatory effect of the ceramide phosphates in exosome membranes as exemplified after second-hand smoke exposure in bronchoalveolar lavage fluid.[82] Lipids are crucial to the activity of EVs, and a thorough examination of EV lipids may reveal new physicochemical features that may be used to distinguish exosomes from other EV subtypes.

3.5 | Surface proteins of exosomes

Antigen presentation on the surfaces of exosomes may regulate immunological responses, making them an important factor in immune system activation and suppression.[83] During the onset and development of inflammation, for instance, exosome membranes fuse with MHC-antigen complexes to trigger antigen-specific T-cell responses.[84] Lymphocyte function-associated antigen 1 (LFA-1) and CD86 are surface receptors on exosomes that signal inflammatory pathways that activate immune cells.[85] Cancer cells that carry the inhibitory checkpoint molecule programmed death-ligand 1 (PDL1) on their surface produce exosomes that reduce the activity of cytotoxic T cells[86] and aid cancer cells in evading the immune system. Exosomes have been shown to transport protein, DNA, and RNA cargoes that may trigger/resolve immunological responses[87] and other physiological processes, in addition to surface proteins.

4 | DELIVERY OF EXOSOME CARGO FROM PARENT TO TARGET CELLS

One recently discovered mode of cell-to-cell communication involves exosomes transporting lipids, nucleic acids, and proteins from parent to daughter cells.[57,88] The exosomal payload, particularly nucleic acids, may control the receiving cell's behavior.[62,89] As an alternative to MSCs, the miRNA payload of exosomes formed from MSCs may aid in the healing of damaged myocardium.[90] Due to changes in miRNA content between exosomes and parental cells, MSC-derived exosomes have demonstrated a higher capacity to avoid hypertrophy than MSCs themselves.[91–93] Several advantages of exosomes have been identified over their stem cell counterparts and discussed later in this article. To modify systemic immunological responses, for example, intercellular communication occurs across long distances thanks to the exosomal transport of hormones, growth factors, and soluble cytokines.[94–96] The lipid payload of exosomes seems to have several roles, including modulating the recipient cells' metabolism and immunological response.[97,98]

5 | CELLULAR SOURCES OF EXOSOMES

Multiple cell types and diverse regulatory mechanisms contribute to exosome production and secretion. Large variations in treatment results have been recorded, and these differences have been linked to the quantity and features of exosomes, including their origin.[99–101] Therefore, rigorous selection of the appropriate cell source of exosomes is crucial for enhancing the yield and quality of exosomes. Furthermore, as no single growth factor is optimal for every single regenerative procedure, no single source of exosomes can be used to treat every disease/disorder. Therefore, research continues to evolve more into precision medicine and discovering which exosomes are best suited for which tissue is of paramount importance.

Most exosomes come from MSCs, which have been well described by the International Society for Cell and Gene Therapy.[102] Menstrual blood, tooth pulp, skin, umbilical cord, adipose tissue, and bone marrow are also potential sources for MSCs.[101,103] Most research has focused on bone marrow mesenchymal stem cells (BMSCs), adipose-derived mesenchymal stem cells (ADSCs), and human umbilical cord-derived mesenchymal stem cells (HUMSCs). As previously established, exosomes generated from each of these stem cell sources may have radically diverse clinical results depending on their contents.[101,103–105] Ongoing research aims to acquire relative data on exosomes from various cell sources, including urine-derived stem cells[106] and dental pulp stem cells,[107] is pivotal toward long-term regenerative therapies of multiple tissue types. Overall, exosomes have been shown to play large roles with the cross-talk of many cell types and have been shown to be implicated in immunity, antimicrobial activity, regeneration of various tissues, while also possessing anti-apoptotic, anti-oxidative stress, and anti-tumor activity (Figure 3).

5.1 | Bone marrow MSC-derived exosomes

Exosomes generated from MSCs in bone marrow (BM) have been examined extensively for their potential to cure a broad range of diseases and disorders. Exosomes generated from human BMSCs have been shown to diminish liver fibrosis by increasing

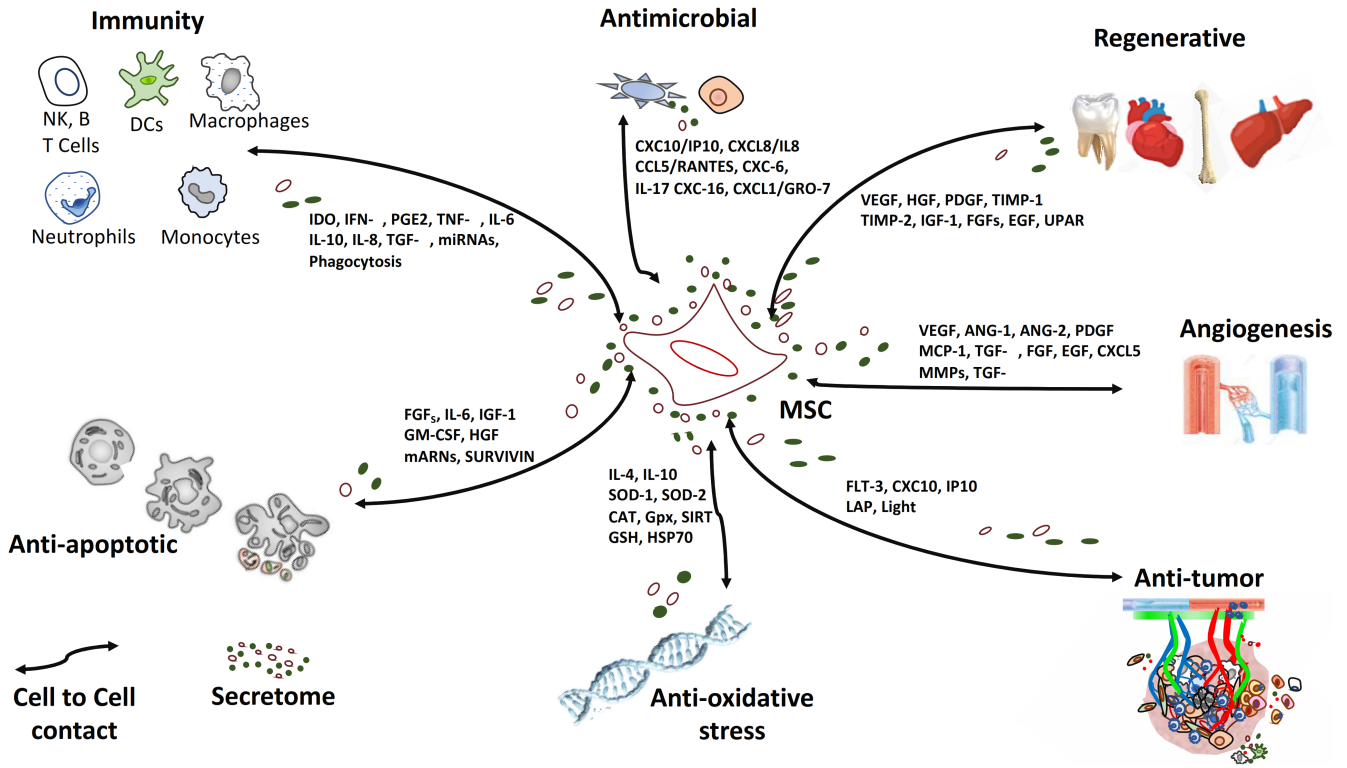

FIGURE 3 Mesenchymal stem cells (MSCs) in the context of a galaxy of intercellular signals. Mesenchymal stem cells participate in different physiological processes through secreted factors (secretome) or by cell-to-cell contact. Reprinted with permission from Fernandez-Francos et al.[301]

hepatocyte regeneration and inhibiting inflammatory processes (as seen by a substantial drop in the expression levels of inflammatory cytokines).[108] Exosomes generated from BMSCs were shown to promote liver regeneration in vivo in another investigation.[109] Multiple studies have also validated the therapeutic potential of BMSC-derived exosomes in various cardiovascular conditions, including ischemic and reperfusion diseases and myocardial damage, and can also protect against myocardial hypertrophy and apoptosis.[110–112]

An array of other studies have further demonstrated the positive outcomes of BMSC-derived exosomes on the recovery process after stroke or/and traumatic brain injury[113,114] as well as degenerative diseases such as Alzheimer's disease.[115] One common feature of BMSC-derived exosomes is their capability to mediate various anti-inflammatory processes.[116] These exosomes have further been tested for rheumatoid arthritis treatment.[117] Bone regeneration experiments using exosomes isolated from BMSCs have also been conducted demonstrating their osteoinductive potential.[118–121]

5.2 | Umbilical cord MSC-derived exosomes

Umbilical cord (UC) MSC-derived exosomes have been utilized and have demonstrated extensive potential in regenerative medicine. UC MSC-derived exosomes have been used to treat liver fibrosis[122–124] and kidney injury.[125,126] Likewise, fracture healing has been improved using locally applied UC MSC-derived exosomes.[127–129] Exosomes generated from UC MSCs have also been found to have beneficial effects in the treatment of gastrointestinal disorders.[130–132]

5.3 | Adipose MSC-derived exosomes

Adipose (AD) MSC-derived exosomes are popular choices since they can be isolated relatively easily when compared to exosomes from other sources. They have a wide variety of therapeutic applications and may speed up the recovery time of damaged tissues. In a porcine model of metabolic syndrome and renal artery stenosis, a single intrarenal delivery of pig AD MSC-derived exosomes reduced renal inflammation, increased the number of reparative macrophages, and upregulated the expression of the anti-inflammatory cytokine IL-10. In addition, exosomes generated from AD MSCs reduced the levels of IL-6, IL-1β, and TNF-α in the renal vein.[133] As a result, these exosomes reduced renal fibrosis and enhanced the functioning of stenotic kidneys.[133]

AD MSC-derived exosomes also provided cardioprotection in numerous studies. In a rat model of acute ischemia/reperfusion, they were shown to prevent myocardial necrosis and apoptosis.[134] AD MSC-derived exosomes also had a protective influence on cardiomyocytes under oxidative stress in vitro.[135]

Exosomes generated from AD MSCs have shown significant promise as a therapeutic for Alzheimer's disease.[136] These authors discovered that exosomes generated from AD MSCs display particular enzyme activity for neprilysin (the most critical enzyme that breaks amyloid beta peptide in the brain).[136]

5.4 | Roles of immune-cell-derived exosomes in diseases

In both the development and treatment of illnesses, immune cells are pivotal players. Exosomes from various cell types carry a wide variety of cargoes to their recipient cells, which might affect their behavior and function. Due to their involvement in many cells' ability to induce inflammatory reactions in response to infection, exosomes have been implicated in a wide range of inflammatory illnesses, including tissue damage, autoimmunity, and allergies. Exosomes may also play a role in improving the regenerative and reparative processes of many diseases.

5.5 | Immune cell-derived exosomes

The activation of other immune cells, inhibition of immunological responses, and participation in the licensing phenomena of antigen-presenting cells are just a few of the many roles that immune cell-derived exosomes (IEXs) may play in the immune system.[137] Below, significant research on each kind of immune cell is examined individually to better understand the properties of the exosomes involved in these processes.

5.6 | Dendritic cell-derived exosomes

Depending on the kind of activation and presence of cytokines, dendritic cells (DCs) release exosomes with varying characteristics. During an inflammatory response, activated DCs release exosomes that stimulate the innate immune system.[138] In addition, exosomes may directly and indirectly stimulate T-cell antigen detection.[139] There is an increase in the number of MHCII peptide complexes on the surface of DCs, which may then activate T cells because of the transfer of antigenic peptides from active to inactivated DCs through DC-specific exosomes.[140,141]

5.7 | Macrophage-derived exosomes

Mass spectrometry analyses have demonstrated that over 5'100 different proteins can exist within macrophage-derived exosomes. Furthermore, their protein content changes after activation and/or polarization. Macrophages are extremely plastic cells with the ability to polarize toward either the M1 (tissue inflammation) or M2 (tissue resolution/regeneration) phenotype. During these events, they release various exosomes that have the potential to act on other cell types.[142–146] Therefore, M2 macrophage-derived exosomes may have a role in controlling tumor cell angiogenesis, invasion, proliferation, and migration.[147–150] These IEXs are expected to have great potential for future use in regenerative medicine since the immune system is highly involved in many diseases.

5.8 | Neutrophil-derived exosomes

Neutrophil exosomes, which vary in composition depending on their activation status, have been analyzed using mass spectrometry, and 271 distinct proteins have been found.[151] Since neutrophils are among the first cells to arrive at the site of inflammation, the exosomes they secrete interact with other cell types and play a crucial role in innate immunity.[152] Neutrophil-derived exosomes, for instance, may change the proliferative characteristics of airway smooth muscle cells and thus contribute to the development of asthma.[151,153]

5.9 | Mast cell-derived exosomes

Depending on the context, exosomes released by mast cells transport exogenous antigens bound to heat shock proteins 60 and 70 (HSP-60 and HSC-70). Exosomes released by mast cells are capable of activating B and T lymphocytes in vivo and in vitro. In addition to their other functions, they may augment DC maturation, making the antigen presentation of DCs more efficient.[154–157] Mast cell exosomes are an intriguing, but further study is needed.

5.10 | Eosinophil-derived exosomes

Exosomes produced by eosinophils exert autocrine effects. The stimulation of nitric oxide (NO) and reactive oxygen species (ROS) generation are among the roles of Eosinophil-derived exosome (EOX).[158] These mediators are less beneficial and relevant than therapeutic exosomes because they promote inflammation by increasing POSTN, CCL26, and TNF-α gene expression.[159]

5.11 | B lymphocyte-derived exosomes

Similarly, activated B cells secrete more exosomes than their inactive counterparts, whether via B-cell receptor (BCR) or TLR activation.[160] These exosomes transport the MHCII peptide complex, which, when bound to T cells, may activate them. Because of the presence of α4β1 integrins that connect with VCAM-1 on the surface of follicular dendritic cells (FDCs), these exosomes may also bind to FDCs.[161] Further studies are required to fully understand the

scope of potential exosomes secreted by B lymphocytes in the repair and regeneration processes.

5.12 | T lymphocyte-derived exosomes

Exosomes released by naive T lymphocytes affect the functions of immune cells due to their expression of the particular TCR and other adhesion molecules.[162] Exosomes expressing markers such as CD73, CTLA-4, and CD25, all of which inhibit the immune system in different ways, are produced and secreted in large quantities by regulatory T cells (Tregs). The exosomal surface protein CD73 induces adenosine synthesis, which dampens the immunological response (anti-inflammatory response).[163] Similar to B lymphocytes, much remains unknown regarding T lymphocyte-derived exosomes. Further study is required to determine how these exosomes impact the functions of other immunological and nonimmune cells.

5.13 | Roles of immune-related exosomes on the prevention of inflammatory responses

IEXs have been used therapeutically due to their potential to modulate immunological responses. Diseases including diabetes, human systemic lupus, and some cancers are linked to an imbalance in Treg/Th17 cells. However, proteins and messenger RNAs (mRNAs) in M2 macrophage exosomes have been shown to have beneficial effects. Zhou et al. found that miR-29a-3p and miR-21-5p are transferred to TCD4+ cells through exosomes from M2 macrophages via a synergistic method. By promoting the development of naive cells into Tregs by blocking the transcription factor STAT3, these exosomes disturb the equilibrium between Tregs and Th17 cells. Anti-inflammatory cytokines such as IL10 are produced in greater quantities, whereas the output of proinflammatory cytokines such as IL4, IL6, and TNF-a is dramatically reduced. Because they promote the development of TCD4+ cells into Tregs, M2-derived exosomes not only stop inflammation but also dampen T-cell anti-tumor responses.[164] Notably, the signaling cargo/content of exosomes released by various immune system cells elicits widely varying responses in target cells.

5.14 | Therapeutic application of IEXs in autoimmune diseases

Damage to normal tissue functions caused by aberrant immune system responses is the hallmark of autoimmune disorders, which may have a devastating impact on a person's quality of life. Due to the growing number of autoimmune-related conditions, exosomes have been evaluated for their ability to modulate immune responses. Exosomes are now widely used and researched for a variety of autoimmune conditions, including myasthenia gravis, inflammatory bowel disease (IBD), rheumatoid arthritis, and multiple sclerosis (MS). Immune-derived exosomes have been useful for improvements in MS,[165–167] IBD,[168,169] RA,[170–172] asthma and allergies,[173–175] and even transplant rejection.[176,177]

5.15 | Astrocyte-derived extracellular vesicles

The central nervous system (CNS) is home to a unique population of glial cells called astrocytes.[178] Inflammation of the nervous system is a recognized pathogenic characteristic in a wide variety of illnesses and disorders; this is known as reactive astrogliosis in the setting of injury. To keep the CNS running smoothly, astrocytes undertake homeostatic roles, including regulating neuronal metabolism and activity,[179,180] and maintaining the glia limitans and the blood–brain barrier (BBB).[181–183] Numerous studies have highlighted astrocyte-derived extracellular vesicles (ADEVs) as a key component of the impact of astrocytes on neighboring tissue. Under both normal and pathological conditions, astrocytes release a variety of substances.

The most significant findings on exosomes derived from various cell sources are summarized in Table 2.

6 | METHODS TO ISOLATE AND CULTURE EXOSOMES

Changes in the components of the culture medium, environmental conditions, or cultivation methods may all have a significant effect on exosome composition and/or production. As a result, these variables may affect the characteristics of exosomes and modify the cells' in vitro productivity. Below is an overview of the methods by which alterations to culture conditions may impact exosome secretion and content.

6.1 | Soluble factors

Adding specific soluble cytokines to the culture medium is a simple technique to manipulate cellular exosome secretion. A myriad of bioactive cytokines, including lipopolysaccharide (LPS),[210] N-methyldopamine,[211] noradrenaline,[211] and adiponectin,[212] have been examined for this purpose. The properties and therapeutic actions of exosomes may undergo dramatic changes under these conditions. Furthermore, each of these soluble factors may have profound effects on the secretion of exosomes and their associated cargo depending on cell type. Therefore, much research is needed in this space to further evaluate how to produce the most significant exosomes for a particular therapeutic application. Notably, however, certain soluble factors have shown the ability to influence exosome secretion by various cell types. Limitations, such as the possibilities of a change in physiological condition or internalization of the agents by the parent cells, mean that there is still a long way to go before

TABLE 2 Examples for recent studies for application of MSC-derived exosomes of various sources in management of multiple diseases.

Sources	Therapeutic mechanism
Adipose-derived MSCs exosomes	• Suppression of neuronal apoptosis and reduces ß-amyloid pathology of Alzheimer's disease (Kamal et al. 2020)[185] • In osteoarthritis: ADMSC-exosomes possess antisenescence effects via downregulation of inflammation and oxidative stress (Tofiño-Vian et al., 2017)[186] • Protection against acute kidney injury through targeting SIRT-1 pathway (Gao et al., 2020)[187] • Promotion of epidermal barrier repair by induction of ceramides synthesis in atopic dermatitis (Shin et al., 2020)[188] • Protection against ischemic brain injury by downregulating miR-21-3p and upregulating MAT2B signaling transduction. (Li et al., 2019)[189] • Attenuation of cardiac damage after myocardial infarction by activating S1P/SK1/S1PR1 and promoting polarization of macrophage M2 (Deng et al., 2019)[190]
Bone marrow MSCs exosomes	• Promoting osteoblast proliferation via MAPK pathway in osteoporosis (Zhao et al., 2018)[191] • Restoring oxygenation, suppression of cytokine storm, and reconstitution of immunity in severe COVID-19 patients (Sengupta et al., 2020)[192] • Stimulation of cutaneous wound healing through TGF- ß /Smad signaling pathway (Jiang et al., 2020)[193] • Promotion of bone regeneration by enhancing angiogenesis (Takeuchi et al., 2019)[194] • Protection against myocardial infarction by promoting autophagy (Zou et al., 2019)[112] • Regeneration of pancreatic beta cells and restoration of insulin in type 1 DM rats (Sabry et al., 2020b) and neurorestorative effects in type 2 DM rats (Venkat et al., 2020)[195] • Improve cognitive impairment, attenuate neurons and astrocytes degeneration, and reduce synaptic loss in diabetic animals (Nakano et al., 2016)[196]
Human embryo MSCs exosomes	• Promotion of osteochondral regeneration and cartilage repair (Zhang et al., 2016)[197] • Alleviation of osteoarthritis by maintaining the normal balance of synthesis and degradation of cartilage extracellular matrix (Wang et al., 2017)[198]
Human endometrium MSCs exosomes	• Enhance cardioprotection in myocardial infarction (Wang et al., 2017)[199]
Umbilical cord	• Suppression of epithelial-mesenchymal transition of hepatocellular carcinoma cells (Xu et al., 2020)[200]
MSCs exosomes	• Tumor inhibition in bladder cancer via delivery of miR-139-5p (Jia et al., 2021)[201] • Repair the heart after myocardial infarction via delivery of circular RNA 0001273 (Li et al., 2020)[202] • Protection against renal interstitial fibrosis via modulating ROS-mediated P38MAPK/ERK signaling pathway (Liu et al., 2020)[203] • Improvement of neurologic function and promoting angiogenesis in spinal cord injury (Zhang et al., 2020)[204] • Inhibition of inflammation and fibrosis and prevention of development of diabetic nephropathy (Xiang et al., 2020)[205] • Increase the sensitivity of ovarian cancer cells to chemotherapy (Qiu et al., 2020)[206] • Traverse the blood–brain barrier, induce autophagy reduce neuronal loss and apoptosis, and increase striatal dopamine repairing Parkinson's disease model (Chen et al., 2020)[207] • Alleviation of acute liver injury through delivery miR-455-3p (Shao et al., 2020)[124] • Promote the recovery of hepatic oxidant injury through the delivery of glutathione peroxidase 1 (GPX1) (Yan et al., 2017)[208] • Decreasing TLR4 expression, NF-$_{K}$B/p65 activation and regulate inflammatory factors expression severe burn-induced inflammation in rats via delivery of miR-181c (Li et al., 2016)[209]

Abbreviations: DM, diabetes mellitus; MSC, mesenchymal stem cell; ROS, reactive oxygen species.
Source: Adapted from Alzhrani et al.[184]

their usage is widespread.[213,214] Furthermore, adding additional by-products and factors poses more regulatory and governmental regulation. Hence, researchers have been reluctant to add soluble factors for large-scale exosome production for clearance purposes by the FDA and CE.

6.2 | Chemical/physical stimulation

Cells undergo phenotypic alterations in response to environmental cues. Thus, various in vitro methods have been formulated to modify and alter cells by various chemical and physical stimulation methods to alter their secretion of exosomes. Stem cells, which operate as tissue producers, have been demonstrated to respond to signals of tissue injury by becoming activated. Researchers have used this theory to justify their efforts to chemically or physically mimic a damaged microenvironment during stem cell development to boost exosome synthesis and thus increase their therapeutic efficacy.[215,216] For instance, chemical stimulation such as hypoxia may affect exosome secretion.[217-219] Serum deprivation is another means to stimulate the secretion of more regenerative exosomes.[220,221]

In addition, bioreactor experiments and radiation have demonstrated that mechanical stresses, including flow and stretching variables, may affect exosome secretion, leading to a 37-fold increase in EV production.[222,223] Ultrasound is another way to increase exosome secretion by 8- to 10-fold.[224]

7 | ISOLATION TECHNIQUES

Exosome processing and separation are dependent on a number of factors, including the initial sample composition. Thus, not only do researchers need to harvest all exosome samples, but much more importantly, the exosomes to be harvested need to be harvested from the correct cell type under the right conditions described above. To ensure long-term viability and high yields, it is crucial to understand the principles upon which the separation process is based, as well as how these aspects impact the quality and features of the products. Exosomes of high purity and yield that are valuable for facilitating life science research and diagnostic and therapeutic applications are the end goal of processing.

Notably, exosomes can be isolated from milk, CSF, saliva, urine, serum, plasma, and cell culture media,[225] and each type requires potentially different processing approaches. Exosomes, plasma proteins, microvesicles, apoptotic bodies, and cell debris are all components of plasma, a complex biological fluid with many components, whose sizes and biochemical characteristics overlap.[226] Due to the lower exosome concentration[227] in urine compared to blood and plasma, more urine is needed to obtain the same yield of exosomes. Exosome mass manufacturing often uses culture medium acquired after cell culture since it is easy, inexpensive, and does not need animal or human subjects to be used.[228] The exosome yield from cell culture medium may be greater than that from plasma or serum.[229,230] Exosome research and development might benefit from a comprehensive study comparing exosome isolation techniques across various sample types.

8 | EXOSOME ISOLATION TECHNIQUES

The six most prevalent methods for preparing exosomes are outlined in Table 3 below. These methods include size-exclusion chromatography, immunoaffinity capture, precipitation, ultrafiltration, and ultracentrifugation. These techniques produce exosomes with varying degrees of purity and yield, and they are often employed in tandem with one another. The physical and chemical foundations of exosome isolation, as well as their methods and commercial applications, are discussed for each method.

8.1 | Ultracentrifugation

The most popular and commonly used method for separating exosomes is ultracentrifugation due to its low price and high efficiency (Figure 4). Because of their different densities, cell debris, apoptotic bodies, and other large components in the culture media may be isolated using this method.[231,232] While high g-forces (100000*g*) have been proven to cause some degree of disruption including EV aggregation, ultracentrifugation in general has a decent recovery rates compared with other approaches.[233,234]

Nevertheless, ultracentrifugation is the most highly utilized process (80% of all studies)[235] and is currently considered the gold standard in the industry.[17] Because of size and density differences, centrifugation at low and high g-forces, as well as additional time, are needed to effectively separate exosomes from other components. At first, the speed at which cells are separated from the cell culture medium is rather slow (300*g*). To remove large cell debris and fragmented organelles, the supernatant is centrifuged at a higher speed (10000–20000*g*). Finally, an even higher speed centrifugation, typically between 100000 and 150000*g*, is used to separate the exosome-containing pellet from the supernatant. Exosome extraction from urine, saliva, cell culture, plasma, and serum are only some of the many applications of this method.[236,237] Exosome isolation typically employs ultracentrifugation rates between 100000 and 210000*g*.[238,239] Separation may be enhanced by increasing the speed, but the downside is that this may run the risk of damaging the exosomes.[240]

8.2 | Size-exclusion chromatography

The second most common method by which to isolate exosomes is size-exclusion chromatography (SEC). Sizewise, exosomes are on the order of tens of nanometers, making them much larger than typical proteins. Columns of porous beads are used to transport liquids. Polymeric beads have pores of varying sizes, allowing molecules with varying radii to pass through them; molecules with smaller radii are forced to move along the column's tunnels and, as a result, elute at a later time. Some molecules, including exosomes, have hydrodynamic radii that are too large to fit through the column's pores and instead move quickly through the medium.[241] As a result, there is less of a need for complex equipment, and sample preparation is straightforward. All sample molecules are then separated in a porous stationary phase whose pore size is determined by the dimensions of the sample.[241] Acoustic fluid separation is a different technique that is reliant on SEC. Here, they are exposed to various acoustic stresses dependent on particle size and may be separated as a result.[242] One of the advantages of SEC is the minimal alterations to the exosome characteristics compared to precipitation-based and ultracentrifugation methods. Therefore, SEC is a very efficient and effective method for isolating exosomes that maintains the biological activity and integrity of the vesicles.[243]

To facilitate exosome isolation by SEC, commercially available prepacked columns are available, such as qEV (Izon Science)[244] and HiLoad Superdex (GE Healthcare).[235] When comparing SEC performed with prepacked columns to EV isolation using precipitation, it was found that SEC yields a lower exosome recovery rate and a more diverse EV population. However, the method may be performed with a freestanding pump and is quick, easy, repeatable, and adaptable to a wide variety of sample types.

8.3 | Ultrafiltration

Ultrafiltration refers the process of isolating exosomes based on size and is characterized by the utilization of very small holes (≈100 nm

TABLE 3 Comparison of common exosome isolation methods and their advantages/disadvantages.

Strategy	Principle	Sample volume	Time (h)	Benefits	Disadvantages	Recovery/ Yield (%)	Purity
Differential Ultracentrifugation	sediment rate	100s of mL	>4	Gold standard, suitable for large-volume samples, relatively cheap	Time-consuming, cumbersome operation, low yield, may damage exosomes	5–20	Medium
Gradient Ultracentrifugation	Density, size and shape	~1 mL	>16	High purity, avoiding exosomal damage	Labor-intensive, preliminary preparation and cumbersome operation	10–40	High
Ultrafiltration	Particles with various size and molecular weight	>100 μL	Generally >4	Easy, without special equipment and reagents	Clogging on filtering membrane, loss of exosomes of small particle diameter	~30	High
Size-exclusion chromatography	Particles with various size and molecular weight	~1 mL	0.3	Simple, economical, maintain the biological function and structure	Special columns and packing are required, lipoprotein contamination	~40–80	High
Immunoaffinity	Surface marker expression	~100 μL	4–20	High specificity for exosome subtypes isolation	Expensive, depending on specificity of the antibody	>90	High
Polymer precipitation	Sedimentation rate	>100 μL	2–18	Simple operation, suitable for large-volume samples	Potential contaminants (co-purifying protein aggregates or residuary polymers)	5–30	Low

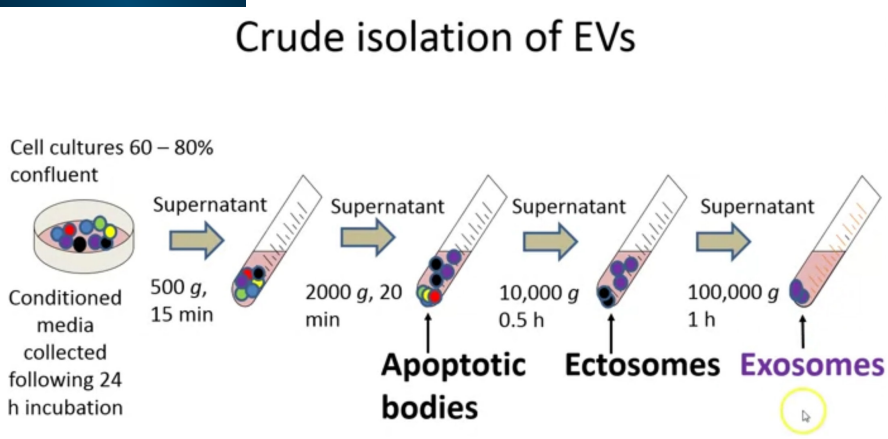

FIGURE 4 Exosome isolation using differential ultracentrifugation. The concept of differential centrifugation is as follows. First, matter with different weights is separated from the sample over time based on density. First, slow centrifugation (500×*g*) is utilized to pellet cells for 15 min. The supernatant is then collected and transferred to a new tube and centrifuged at 2000×*g* for 20 min to pellet apoptotic bodies. Thereafter, the supernatant is collected and centrifuged at 10 000×*g* for 30 min to pellet the exosomes. Finally, the supernatant is collected and centrifuged at 100 000×*g* for 1 h. Exosomes are then collected from the remaining pellet.

diameter).[245,246] Ultrafiltration processes have a high-throughput since a single filtration cycle takes just seconds to 30 min. Ultrafiltration is a method of isolating vesicles by forcing sample fluid through a membrane with holes smaller than 100 nm.[32] Additional processes using membranes with smaller or larger pore sizes may be utilized to filter out even more impurities. The shear stress caused by the applied pressure is the technique's greatest drawback, as it might cause the exosomes to become damaged or lost as a consequence of membrane adherence and membrane obstruction caused by the buildup of particles.[32] Tangential, centrifugal, tandem, and sequential flow filtration are examples of exosome ultrafiltration techniques.[32,247]

8.4 | Polymer-mediated precipitation

The hydrophilic polymer polyethylene glycol (PEG) is used in polymer-mediated precipitation to isolate viruses from biomacromolecules. More than fifty years have passed since the method's inception.[248] The polymer traps water molecules, decreasing exosome solubility and allowing them to settle under low-speed centrifugation. To precipitate exosomes, exosome-containing samples are treated with a PEG (Mol. Wt.: 8000 Da) solution. This precipitate might be separated and reprocessed by centrifugation or filtering after incubation at 4°C overnight. There are a number of commercially available exosome isolation kits, including PureExo®, ExoQuick®, Exo-spin®, and miRCURY®. These kits induce exosome precipitation using polymeric additives (for use with specific reagents) and allow for separation to occur in 30 min using a traditional centrifuge (10 000 *g*).

8.5 | Precipitation

Exosome characterization often employs precipitation techniques. In studies conducted all across the globe, precipitation was found to be the method of choice for analyzing EV RNA.[249] Polymers that have a high molecular weight but low water solubility are used in precipitation techniques to bind water molecules and precipitate insoluble substances.[248] Biological components are concentrated until their solubility is surpassed, at which point they precipitate out of solution. The lower product purity obtained with this method means that this approach has restricted therapeutic utility in trials, despite its higher yield.[250]

8.6 | Immunoaffinity-based capture

Isolating exosomes from bodily fluids has been made possible by Immunoaffinity-based capture (IAC), which makes use of the binding affinity of proteins to protein receptors found in the membranes of exosomes and vice versa. Using exosome indicators such as Tim-4 binding to phosphatidylserine, CD326, and CD63, ELISA is a typical approach that uses IAC to collect and quantify exosomes.[232] IAC may also be used to further refine the purity of exosomes separated by density- and size-based methods. Isolating tumor-specific exosomes is a time-consuming process due to their low abundance. It was proven that by using mAb 763.74, which is specific for the CSPG4 epitope produced by certain cells, the IAC-based technique could separate and extract melanoma-specific exosomes. This technique was shown to be effective for liquid biopsy, with a capture rate of approximately 95% for exosomes that are unique to melanomas.[251] However, this method is useless because although it is efficient, it is not practical for therapeutic purposes, as many biological materials are added/incorporated during the exosome isolation process. Therefore, IAC methods are typically utilized for diagnostic purposes.[252]

8.7 | Microfluidics-based technologies

Exosome isolation and detection may be performed quickly and efficiently using microfluidics. Exosome separation techniques use

microfilters, nanoarrays, and nanowires, all of which are small enough to isolate exosomes. First, silicone nanowires are carved on the sides of micropillars to serve as traps for liposomes. The second technique involves utilizing microbeads to perform acoustic nanofiltration, which isolates exosomes with a size in the range of 100–1000 nm. Particles of varying sizes may be separated using viscoelastic microfluidics, in which a viscoelastic medium, such as poly(oxyethylene) (PEO), is subject to elastic lift forces. This method has the potential to achieve separation efficiencies of 80% and purities of 90%.[253] This method is preferred for exosomes diagnostics versus isolation for therapeutic use.

Other techniques exist, including dielectrophoretic segregation (DEP) and tangential flow filtration (TFF), although these methods are much less frequently utilized.

9 | QUANTIFICATION OF EXOSOMES

9.1 | Methods of quantification

Due to their diminutive size, conventional molecular biology measurement techniques are unreliable when used with exosomes. Improved technology and instruments have led to a variety of approaches being used for their quantification at present. Current quantification techniques, their underlying concepts, and their benefits and drawbacks are summarized in Figure 5 and Table 4 and discussed below.

9.2 | Nanoparticle tracking analysis

Using the variations in the light scattered by suspended particles owing to their Brownian motion, the particle concentration may be determined using Nanoparticle tracking analysis (NTA), which has been utilized for the measurement of exosomes.[254,255] Particles in suspension in a sample chamber are illuminated by a laser beam, and a video of the particles in Brownian motion and scattered light is captured by a light-sensitive CCD camera placed atop a long working distance microscope.

The Stokes–Einstein equation is then used by an external program to analyze the video, isolate the individual particles, and determine their hydrodynamic radii. The sample's particle concentration may then be calculated by counting all of the particles inside the camera's field of vision, with the result expressed in terms of particles per cubic centimeter. Since NTA does not alter the material in any way and may be used to identify and quantify exosomes, it is the technique of choice for this purpose. Other EVs in solution will not be identified when using fluorescent mode, which is designed to detect only fluorescently labeled exosomes.[256] The instrument's size restriction of 30-500 nm means that the concentration of EVs larger than 500 nm will be underestimated.[257]

Reproducible findings need both the utilization of expensive instrumentation and an expert understanding of software and hardware configurations. In addition, it is possible that photobleaching and background signals from dye aggregates can muddy the findings. When gauging EVs, NTA is often used. Lipoproteins of comparable

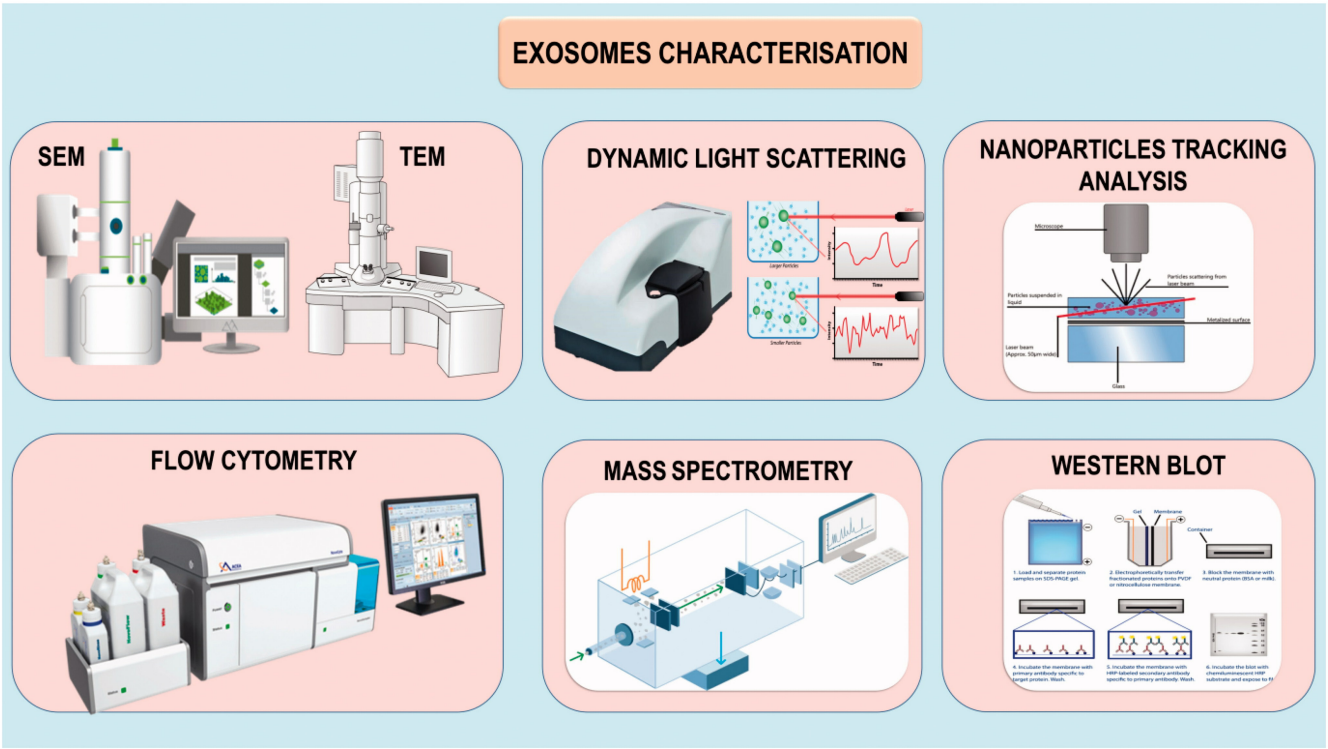

FIGURE 5 Methods for exosome characterization. Reprinted with permission from Modani et al.[338]

TABLE 4 Methods of exosome quantification.

Method	Principle	Advantages	Disadvantages
Nanoparticle tracking analysis (NTA)	Based on the detection of light scatter by particles in suspension and their Brownian motion to estimate the number and volume distribution of EVs	Does not rely on detection of a specific marker Direct quantification	Expensive instrument Photobleaching and potential background from dye aggregates Measures non-exosomal contaminants also
Flow cytometry	Flow cytometry detects particles suspended in a fluid by their interaction with a laser beam as they flow through a detection cell	Direct quantification	Insensitivity to smaller exosomes. Requires binding to fluorophore-conjugated antibody-coated beads Swarm effect that means multiple smaller vesicles are counted as single particle. This may provide false positive result
Tunable resistive pulse sensing	Detects the passage of individual particles through a pore in a membrane	Direct quantification	Pore clogging. Insensitivity to smaller exosomes. Measures non-exosomal contaminants also
Electron microscopy	Imaging of individual exosomes under scanning electron microscope	Exosomes are manually counted	Labor-intensive, slow process
Dynamic light scattering	Evaluates fluctuations in the light scattering intensity of particles	High sensitivity, simple sample preparation, rapid	Heterogeneous exosome populations cannot be analyzed, difficulty with polydisperse samples
Microfluidics- based detection	Transport of fluids controlled by capillary forces	Product purity, high-throughput analysis	Not ready for industrialization yet, increase in the signal/noise ratio is encountered
Surface plasmon resonance (SPR)	A light is focused to a metal film through a prism and the reflected light is detected which is collective oscillation of free electrons. It is sensitive to change in refractive index of the media	Label-free and real-time quantitative analysis technique High sensitivity of up to l nM for a 20 kDa protein Specific to the binding event	Difficult to discriminate between specific and non-specific interactions Mass sensitive limitation Limited sensor area Expensive instrument and sensor cost
Single particle interferometric reflectance imaging sensor (SP-IRIS)	A monochromatic light illuminates on sensor surface and scattering signal from individual nanovesicles is detected by *CMOS* camera The signal is enhanced due to the interferometric phenomena	Quantitative, label-free, and dynamic detection method Multiplexed phenotyping and digital counting of individual EVs with diameters of 50–200 nm	Detection limit of 3.94E+09 particles/mL

sizes, however, may skew the findings of EV measurements. Users are encouraged to analyze the data correctly and rigorously adhere to established guidelines.[258]

9.3 | Flow cytometry

Many laboratories and institutions have access to a flow cytometer, making it the instrument of choice for analyzing exosomes. This is because flow cytometers can analyze many parameters simultaneously. Due to size detection restrictions, traditional cytometers may fail to pick up particles smaller than 300 nm. Particle resolution might be improved with the use of multiangled lasers if the latest generation of flow cytometers are updated.[259]

Apoptotic bodies and microvesicles may both be detected by flow cytometry since this technique can routinely and relatively simply count particles larger than 500 nm.[260] Although exosomes are too small to be detected by a flow cytometer on their own, they may be identified when they are bound to antibodies against antigens on the exosome membrane. After being attached to secondary fluorophore-conjugated antibodies, the counting beads are suspended in fluid and sent through the center of a detection cell as a single particle stream under the guidance of the sheath fluid. An incident laser beam excites the fluorophores bound to the beads, causing them to fluoresce and emit longer wavelength light. The fluorescence detector then detects the fluorescence intensity and counts the number of emission "events" to determine the concentration of beads and, by extension, exosomes in the sample.[261] Particle-scattered light will also be detected and quantified, as signals from forward scatter will be picked up by a detector placed in front of the laser beam, while those from side scatter will be picked up by a detector placed on the side of the beam. The warm effect is one of the major obstacles in exosome quantification using flow cytometry. When a cluster of very small vesicles is recorded as a single

event, misleading conclusions might be drawn. This happens when the scattering or fluorescence signal exceeds the detection limit because of a large concentration of very small vesicles in the sample.[262]

9.4 | Tunable resistive pulse sensing

Tunable resistive pulse sensing (TRPS) uses voltage delivered across a membrane's pores to identify specific particles.[263] The pore size may be adjusted to accommodate a wide range of sample dimensions. When a sample of exosomes is placed at one end of a membrane and a single exosome is pushed through the pore, the increased resistance within the pore causes the current going through the pore to decrease. The quantity and size of particles passing through the pore may be determined by monitoring and analyzing the variations in the current. An "event" is defined as a reduction in the current of one microampere, and the number of events is proportional to the exosome concentration in the sample.[263] This technology has certain drawbacks, such as the possibility of pore clogging with repeated usage of the membrane and the insensitivity of the equipment, which does not detect very small exosomes.[264] Owing to the requirement for more specialized equipment, it is not a commonly employed technique in the field of exosome detection and quantification.

9.5 | Electron microscopy

The most popular technique for verifying the purity of isolated exosomes and ensuring that the vesicles are not damaged is electron microscopy. The imaging and morphological display method known as whole-mount negative staining is often employed for exosomes. However, cryo-TEM imaging has shown that exosomes are entirely spherical, and the cup form is a byproduct of the drying process necessary to prepare the specimen for imaging. Exosome morphology may be preserved with blocking sectioning, plastic embedding, and fixation with glutaraldehyde, which may be chosen for observing the native structure of extracellular vesicles.[265,266] Electron microscopy is still helpful for verifying the exosome isolate's shape and purity, but it is too time-consuming and has too low throughput to be used for accurate counting. Due to vesicle loss during sample preparation, electron microscopy is also likely to underestimate the true number of exosomes present.[267] Nevertheless, its use has been widely employed for exosome characterization and quality assessments following isolation and less utilized for quantification.

9.6 | Dynamic light scattering

Dynamic light scattering (DLS) is a widely used method for quantifying nanoparticle size. Particles in a solution undergo Brownian motion, which causes them to move randomly and collide with one another, exchanging energy in the process. Smaller particles are more affected by the energy transfer since they go through the solvent at a greater velocity. Particles in solution will disperse a beam of incoming light in all directions.[268] Particle size and concentration in a solution may be determined by monitoring the intensity in the variations in scattered light over time at a certain angle using a fast photon detector to learn more about the particles' motion. The intensity of the dispersed light will be more volatile over time when smaller, faster-moving particles are involved. The particle diffusion coefficient (D) and intensity correlation function (R) are examined to learn more about the fluctuations. The size of the particles in the solution may be calculated using the Stokes–Einstein equation, which establishes a relationship between particle density ('D') and particle radius ('R'). If the polydispersity of the solution is less than 0.1, all of the particles in the sample are the same size, which may be determined using DLS.[269] The time-dependent variations in the amount of light dispersed by the moving particles are measured and recorded. Although DLS is a versatile tool for quantifying exosomes, it is seldom used in practice.

9.7 | Microfluidics

In microfluidics, micron-sized channels are utilized to manage fluid volumes on the order of microliters to milliliters. Exosomes in cell culture media or a patient sample may be immunocaptured, quantified, and characterized using a microfluidic device. Microfluidic devices were employed by Fang et al. to detect TEM-characterized exosomes. Using a programmed pump system, the immune-captured exosomes were measured by determining their capture efficiency, which remained constant on chip.[270] This method of quantification is practical due to its minimal sample size requirements, low cost, and straightforward procedures.[271] This method's great sensitivity indicated that miRNA might be analyzed and quantified for use in clinical care and biological research.[272]

9.8 | Surface plasmon resonance

Much research has been conducted on Surface plasmon resonance (SPR) and its uses in the last decade. Resonant electron oscillations caused by a refractive index mismatch between a material's surface and incoming light are used for SPR, a label-free detection method.[273,274] SPR has been shown to be a solid method for investigating biomolecular interactions. In addition, SPR may be used to identify and quantify exosomes. For instance, an SPR sensor probed with anti-CD63 antibodies was used to measure the concentration of exosomes containing the tetraspanin membrane protein CD63 by converting the SPR response into a surface-bound mass. It was found that the margin of error for the measurements was approximately ±50%.[275] Nevertheless, other methods have proven to be simpler with better detection abilities.

9.9 | Single-particle interferometric reflectance imaging sensor

Multiplex phenotyping and digital counting of distinct populations of exosomes larger than 50 nm utilizing microarray-based solid-phase chips is possible with the Single-particle interferometric reflectance imaging sensor (SP-IRIS) technique. After bouncing off the SiO2 layer, the IRIS signal is affected by interference from the fields. IRIS is dual-modal in that it can both measure biomass with high-throughput and digitally identify single particles at high magnification without the need for labels.[276] There are a variety of methods for analysis and characterization, although some of them may lack sensitivity due to the small size of exosomes.

10 | EXOSOME CHARACTERIZATION

The quantity and purity of biomolecules, including nucleic acids, lipids, and proteins, may be evaluated together with the overall quality of the isolated exosomes using quantitative characterization techniques.[277,278] Several parameters are important, as discussed below.

10.1 | Total exosome count

Methods that count particles are used to calculate exosome yield,[279] including electron microscopy (EM), resistive pulse sensing (RPS), dynamic light scattering (DLS), fluorescence correlation spectroscopy (FCS), flow cytometry, and nanoparticle tracking analysis.[256,280–282] FCS, flow cytometry, and NTA are the most commonly used methods. DLS and RPS tend to overstate the overall number of particles, making them unreliable for counting.[279]

10.2 | Protein content

Exosome purity may be evaluated, in part, by determining the amount of protein they contain (in terms of mass). The purity of a sample may be calculated as the percentage of protein mass relative to the total number of exosome particles.[279] Protein markers are identified and quantified with the use of enzyme-linked immunosorbent assays (ELISAs) and mass spectrometry.

The mass-to-charge ratio of ions is detected in mass spectrometry, a high-throughput method for compound detection. To prevent exosome degradation during analysis, mass spectrometry methods employed in exosome proteomics[283] need only little sample pretreatment.[284] Exosome-specific proteins may be systematically characterized using mass spectrometry and bioinformatics.[285]

ELISA is a standard method for quantitatively analyzing proteins and peptides via the use of antibodies.[283] The detection of protein markers and the quantification of tumor antigens on exosomes and exosome-specific antigens are possible thanks to ELISA, which has been employed for exosome profiling and diagnosis.[259] Some protein indicators may be inexpensively analyzed using ELISA, while protein quantification in complicated biological samples can be accomplished with mass spectrometry.[286]

10.3 | Lipid composition

Exosomes may be categorized into several classes based on the lipids they transport in their membranes. Targeted exosome purity may be measured by calculating the percentage of total exosomes that contain a target lipid.[279] Lipid quantification methods include Fourier transform infrared (FT-IR) spectroscopy, fluorescence microscopy, and sulfo-phospho-vanillin (SPV) assays.[287,288] Lipid concentrations are determined by SPV assays, in which a colorful product is formed from the reaction between phosphovanillin and the carbonium ions obtained from lipids in the presence of sulfuric acid.[288,289] For reliable findings, sample concentrations over 50 μg mL1 lipid are needed.[289] Lipids in exosomes can be quantified using fluorescence microscopy in combination with a lipophilic dye for plasma membranes, such as PKH26 or DiR, and comparison of exosome images to reference standards.[290–292] Compared with SPV and fluorescence microscopy, FT-IR gives lipid counts with greater precision, consistency, and speed with a lower cost and sample volume.[289,292] However, FT-IR is insensitive to cholesterol and other sterols because their C-C and C-H vibrational bands are easily confused with those of other compounds.[289]

10.4 | DNA/RNA analysis

DNA and RNA may be transported between cells through exosomes. Exosome purity may also be evaluated by calculating the ratio of targeted DNA/RNA sequences to the total amount of exosomes. Microarrays, next-generation sequencing (NGS), and polymerase chain reaction are only a few of the common nucleic acid quantification methods that may be used for exosome DNA/RNA research.[293–296] When compared to NGS and microarray technologies, PCR is considered the gold standard because of its superior sensitivity and accuracy.[297]

11 | DISCUSSION

This first article in this two-part series was designed to promote a better understanding of exosomes, including their sourcing, isolation techniques, quantification, and characterization. Exosomes offer many advantages as regenerative biomolecules, and their therapeutic potential is discussed in the subsequent article. Below, we highlight some relevant discussion points.

11.1 | Advantages of exosomes over cell-based therapies

One area that has gained much research interest the comparison of exosome therapy to cell-based therapies such as stem cell therapy. Exosomes offer many advantages in this regard with 3 of their main advantages being 1) exosomes will never initiate immune refusal, which has been one of the main limitations of stem cells, 2) exosomes have superior suitability for storage and ease of use, and 3) exosomes are small enough to cross over many small barriers, such as the blood–brain barriers, whereas cells are not.

Stem cell-derived exosomes, for instance, offer the possibility to promote cell differentiation and angiogenesis and improve cell survival/activity. In addition, exosomes may quickly foster better microenvironments by dampening inflammatory reactions. Cell-dependent treatments have been criticized for their slow response time, which is especially problematic for those who need immediate medical attention, such as those suffering from myocardial infarction. For instance, when injecting stem cells intravenously, many MSCs enter into circulation, which can very well lead to entrapment within the lungs. However, exosomes, because of their small size, are able to bypass the lung and enter the bloodstream where they may more precisely reach their intended target tissues. Encouragingly, the inner exosomal composition may be greatly modified by cell sourcing, laboratory setting, mechanical stimulation, etc. Thus, many possibilities exist.

Researchers and authors have recently revealed that exosome-based treatments are more promising than stem cell-based therapies because of their more well-defined processes.[298] The bilayer architectures and chemokine, cytokine, microRNA, mRNA, and immunomodulatory substance components endow stem cell-derived exosomes with excellent pharmacokinetic properties, biocompatibility, and tissue-targeting capacity.[221,299–301] In addition, several studies have shown that exosomes can lower inflammation, control cell proliferation, and speed up the recovery of injured tissues.[300,302] Exosomes have been shown to improve the skin,[303] muscle and bone,[304–306] nerve,[307] heart,[308] liver,[309] kidney,[310] lung,[311] immune system,[312] and virus infection.[313] Moreover, the ability of exosomes to be utilized for both the detection and resolution of tumorigenicity and immune rejection associated with cell therapy, along with the convenience and ease of obtaining exosomes without the complexities of cell isolation and potential damage, have further expanded the design and clinical application of exosome-based therapies.[299,314]

In summary, the therapeutic potential of MSC-derived exosomes favoring tissue repair and their clinical use may offer significant advantages over their live cell counterparts. This is because they may produce fewer unwanted side effects, such as infusional toxicity, uncontrolled cell growth, and the possibility of tumor formation, all of which have been debated for over 20 years with stem cell-based therapies. Exosomes cannot change or copy themselves, and they cannot spread. They have been tested in various animal models for various human diseases (e.g., hypoxic pulmonary hypertension,[315] acute kidney injury,[316] and liver fibrosis[122]), where it was determined that their functions are either equivalent to or better than those of MSCs, all while being much safer.

Previously, it was reported that exosomes are preferred over cell therapy for five reasons.

1. These vesicles can be kept for a long time without losing their ability to boost the defense system.
2. Exosomes can better integrate with the target cell than soluble factors made by cells because their surfaces look like those of body cells.[317]
3. Since they are much smaller than cells, exosomes may travel freely across capillaries and various barriers such as the blood–brain barrier.[318]
4. Exosomes can be readily manipulated and engineered.[319]
5. Injection of exosomes is easier than that of cell therapy (and there is the possibility of nasal administration).[319]

11.2 | Drug delivery

The blood–brain barrier and the matrix of highly structured tumors are two examples of biological barriers that are notoriously difficult for therapeutics to penetrate. However, exosomes may offer a natural platform for improved targeting and functional transfer of therapeutics across these barriers. As a result, there is much enthusiasm about using exosomes for cutting-edge drug delivery methods to treat a wide variety of complex diseases. To date, incubating parent cells with desired molecular cargo has allowed for the passive loading of specialized cargo.[320] While this line of research remains in its infancy, it may present superior ways to deliver therapeutic drugs to target tissues while avoiding immune clearance.[321]

11.3 | Cellular origin and exosome isolation

The cellular origin of exosomes is a key component in determining their biodistribution and composition. Exosomes from different cellular sources may carry dramatically different cargo,[322] and subsequent studies have shown that there is great variability in therapeutic outcomes. Therefore, much like one growth factor cannot fulfill the roles and requirements to regenerate all tissues, no single exosome or exosome source can fulfill the roles to regenerate all tissue types. Therefore, additional research needs to specifically address which exosome, which cell source, and which culture conditions or isolation technique are most effective for the therapeutic application of exosomes for the treatment of various individual diseases. This will lead to more favorable personalized medicine once a better understanding of exosomes is accumulated.

11.4 | Characterization and standardization for the future

One of the downfalls in the advancement of exosome research has been the rather poor reproducibility and lack of proper documentation during various research endeavors. To improve reproducibility, critical interpretation, and transparency, it is strongly advised that all authors publishing in this field follow the most up-to-date MISEV guidelines and provide full disclosure on the methods used for particle isolation and quantification.[323] Greater cooperation within the EV community is needed to produce a set of standard resources and guidelines in light of the lack of uniformity in EV loading studies and the high degree of diversity in methods utilized across the literature.[323] The International Society for Extracellular Vesicles (ISEV) was founded in 2011 with the goal of encouraging standards in this area. In 2014, the first position statement on the minimum information for studies of extracellular vesicles (MISEV) was issued.[324]

11.5 | Optimization for storage

Another commonly asked question is how to store exosomes for maximum potency. The International Society for Extracellular Vesicles recommends storing exosomes in phosphate-buffered saline at -80°C.[231] Storage at temperatures over -80°C reduces the number and content of exosomes, whereas storage at lower temperatures has less of an effect.[325,326] It has also been demonstrated that exosomes can be better preserved from cryodamage when trehalose and certain preservative agents, such as DMSO and sucrose, are added.[327] Research is ongoing as to whether lyophilization is possible in the future.

11.6 | Challenges with bringing exosomes into everyday clinical practice

There is substantial scientific evidence regarding the therapeutic efficacy of exosomes for a variety of treatments of various human disorders. Their success, however, resides in their production rather than their application in medicine. To create therapeutic exosomes acceptable for clinical use, it is necessary to improve CMC (chemical, manufacturing, and control) development for good manufacturing practices (GMP). Creating a master cell bank (MCB), developing a methodology for large-scale exosome synthesis/isolation, and creating quality control (QC)/analytical methods for therapeutic exosome manufacturing are all part of CMC development for exosome therapies.[328–331] Because exosomes are such a new therapeutic platform, no one has yet devised a universally accepted procedure for their CMC.

To produce exosomes on either a small or large-scale, various cell types have been cultured in fixed or moving culture systems such as flasks and bioreactors.[332–335] Thus, a robust and efficient isolation and purification process is crucial to obtain a high yield of pure exosomes.[336] Since many research reports have demonstrated variability in various isolation techniques and differences in exosome isolation, there remains a significant need for technical improvements. This, along with the high costs of commercialization and the lack of insurance coverage and government bodies regulating exosomes with FDA/CE clearance, has drastically slowed the rate toward widespread commercialization.

DATA AVAILABILITY STATEMENT

Data available on request from the authors

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