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Review

Potential Avenues for Exosomal Isolation and Detection Methods to Enhance Small-Cell Lung Cancer Analysis

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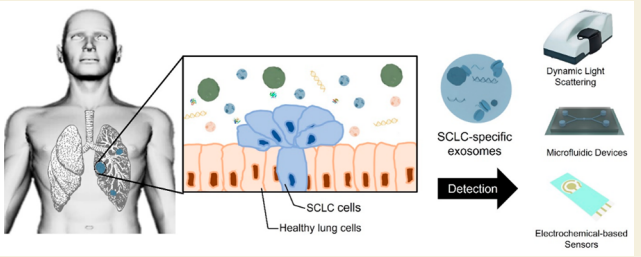
ABSTRACT: Around the world, lung cancer has long been the main factor in cancer-related deaths, with small-cell lung cancer (SCLC) being the deadliest form of lung cancer. Cancer cell-derived exosomes and exosomal miRNAs are considered promising biomarkers for diagnosing and prognosis of various diseases, including SCLC. Due to the rapidity of SCLC metastasis, early detection and diagnosis can offer better diagnosis and prognosis and therefore increase the patient's chances of survival. Over the past several years, many methodologies have been developed for analyzing non-SCLC-derived exosomes. However, minimal advances have been made in SCLC-derived exosome analysis methodologies. This Review discusses the epidemiology and prominent biomarkers of SCLC. Followed by a discussion about the effective strategies for isolating and detecting SCLC-derived exosomes and exosomal miRNA, highlighting the critical challenges and limitations of current methodologies. Finally, an overview is provided detailing future perspectives for exosome-based SCLC research.



KEYWORDS: *small-cell lung cancer, exosomes, exosomes-derived miRNA, diagnosis, liquid biopsy, nanotechnology based assays, microvesicles, characterization, microfluidics, biomarkers*

1. INTRODUCTION

Lung cancer is the leading cause of cancer mortality globally, with approximately 2.1 million new cases and 1.8 million deaths in 2018.[1] The two primary malignant tumors of the lungs are non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). NSCLC is the leading cause of death worldwide, accounting for 85% of all lung cancer cases, while SCLC accounts for 15% of lung cancer cases.[2] SCLC is a high-grade aggressive neuroendocrine carcinoma with few effective treatment choices. SLCS's early metastasis results in a poor prognosis and, as a result, frighteningly low overall survival rates. Thus, identifying distinctive markers to improve the early detection of SCLC could redefine this disease's diagnostic and prognostic landscape.[3]

Circulating tumor cells (CTCs), circulating tumor-specific nucleic acids (ctDNA, ctRNA, miRNAs, lncRNAs), extracellular vesicles (EVs) (e.g., exosomes and apoptotic bodies), and autoantibodies are some of the most prevalent circulating biomarkers (CBs) with strong diagnostic, prognostic, and therapeutic potential.[4] The study of CBs in SCLC may open new avenues for monitoring the molecular phenotype of a patient's tumor during the disease and identifying biomarkers for tumor progression and ministration.[5] Among these CBs, tumor-derived exosomes (TDEs) have become potential biomarkers for the early diagnosis of various cancers, including SCLC. They have been used as a biomarker for examining cancer heterogeneity, tracking cancer patients after treatment, monitoring the resistance development to therapy, and contributing to the precise and personalized treatment of SCLC patients.[6] A critical significance of TDE-based studies is that since SCLC treatment does not include surgery, it is challenging to isolate tissue samples. They can, however, be easily isolated from the body fluids and could serve as promising "liquid biopsy" biomarkers of SCLC. Additionally, these TDEs contain proteins, lipids, and nucleic acids that resemble the molecular profiles of the originating parental cancer cells, thus making these TDEs a promising tool for investigating SCLC in a clinical setting.[7] Moreover, TDEs-derived exosomal miRNAs appear to be a gene signature that could reveal information about the pathobiology and prognosis of the disease.[8] With exosomal miRNA transfer between cancer and stromal cells being linked to the development and spread of cancer in the tumor microenvironment, including lung

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cancer,[9] it is more apparent that using miRNAs as SCLC biomarkers for early detection and diagnosis has the latent potential to improve the course of treatment, which is critical in a clinical setting. Exosomal microRNAs are associated with numerous pathophysiological processes in SCLC, including epithelial-mesenchymal transition, growth, proliferation, migration, invasion, and angiogenesis, all of which ultimately result in tumor progression and metastasis.[10] Furthermore, the absence of biomarkers for treatment selection and monitoring patients with SCLC and their therapeutic options lead to poor outcomes, making new prognostic biomarkers to enhance their management. Given the invasive nature of diagnosing SCLC, developing alternative approaches, such as detecting molecular markers such as exosomal miRNA that are linked with this disease, may enhance the diagnostic and prognostic efficacy.[11] Additionally, introducing non or minimally invasive diagnostic tools could be rolled out for at-risk individuals to diagnosis patients at earlier stages of the disease allowing for increased prognosis.

Over the past decade, several exosome separation methods have been developed and have exhibited promising results, with a dominant focus on the exosome's biochemical and physiochemical properties. The notable techniques include ultracentrifugation- (differential and density gradient), particle size- (i.e., ultrafiltration and size exclusion chromatography), immunoaffinity-, polymer-, and microfluidics-based platforms. These techniques isolate all the sample's exosomes, including exosomes from different cell types, such as carcinoma cells. After isolating and purifying all the exosomes, it is essential to accurately characterize and quantify the TDEs, primarily for diagnostic purposes. Some of the most popular methods for the characterization and quantification of exosomes include dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), atomic force microscopy (AFM) imaging, and electron microscopy (EM) analysis.[12] These methods have a "fundamental limitation"; they cannot distinguish (i) exosomes from other nanoparticles of similar size and (ii) TDEs from total exosome populations. To quantify all the exosomes present in the samples, universal exosomal membrane markers, such as CD9, CD63, and CD81, have been widely used. These markers are widely expressed in exosomes released by almost all cell types. However, to quantify TDEs within the total population of exosomes, cancer-specific markers in combination with the negative control protein biomarkers have extensively been utilized for better characterization. Western blot and ELISA commonly detect these protein biomarkers. Fortunately, thanks to the quick development of microfluidics detection techniques, high throughput analysis may be carried out with outstanding precision and little reagent consumption. Examples of microfluidics-based exosomal proteins detection techniques include fluorescence correlation microscopy (FCM), colorimetric detection, surface plasmon resonance (SPR), nuclear magnetic resonance (NMR), and electromagnetic detection.[13]

For the detection of exosomal miRNAs, the exosomes must be purified. Therefore, ultracentrifugation,[14] commercially available isolation kits such as the Total Exosome Isolation Kit, and polymer-based ExoQuick reagent[15] are the traditional isolation techniques. Once isolated, Northern blot,[16] quantitative reverse transcription polymerase chain reaction (qRT-PCR), and next-generation sequencing (NGS) are the most commonly used methods for detecting exosomal miRNAs, including those expressed in SCLC. Several efficient and sensitive methods for exosomal miRNA detection have also been developed based on the aptasensor, enzymatic, and nonenzymatic isothermal amplification methods, including cyclic enzymatic amplification hybridization chain reaction, catalytic hairpin assembly triggered DNA walker, and rolling circle amplification (RCA)-assisted CRISPR/Cas9 cleavage (RACE).[17,18]

Exosomes and exosomal-miRNA analysis methods have many challenges, with the enrichment of exosomal subpopulations being technically complicated. As antibodies that work effectively with tissues or pure proteins may not work well with exosomes due to the orientation and/or folding of the surface protein in the membrane or the availability of the epitope on the exosomal surface, well-established methods are required. Exosomes' surface features allow them to adhere to various surfaces, including other exosomes. Nonspecific binding to the extraction tube or bead surfaces during purification processes might result in biological material loss and decreased specificity.[19]

Furthermore, isolation efficiency depends on the quality of the sample, resulting in variable isolation yields.[6] Existing separation and isolation techniques yield insufficient quantities of exosomes and are expensive for large-scale production.[13] Additionally, there are still obstacles in quantifying and detecting exosomes. Although numerous methods have been developed for phenotyping and quantifying exosomes, the need for a consensus regarding detecting these vesicles has resulted in substantial controversy and contrasting findings. Due to the heterogeneity of exosomes, the low refractive index, and ineffective methods for determining the particles' size range, the assessment of these vesicles has been called into question.[6] Some cargo compositions, like miRNAs, appear to be affected by isolation approaches. Rekker et al.[20] concluded that isolation procedures could influence the exosomal miRNA profile, leading to contamination; the isolated exosome fractions are frequently "contaminated" by the coisolated plasma proteins.

Exosomes and exosomal miRNA can only be validated as diagnostic, prognostic, or therapeutic biomarkers once a well-recognized method is introduced for their characterization. While cutting-edge technologies are being introduced and evaluated to address it, the outcomes of this research are still vulnerable to criticism.[21] This Review discusses the current advances in the isolation and detection of lung and SCLC -derived exosomes and exosomal miRNA, highlighting significant biological and technical challenges associated with these methodologies. Future perspectives for enhancing exosomes and exosomal miRNA-based SCLC diagnostics research are also provided.

2. BIOGENESIS OF EXOSOMES

The endosomal sorting complexes required for transport proteins (ESCRT) are the best-described mechanism for the biogenesis of exosomes (Figure 1). Briefly, this system involves the formation of intracellular endosomes through internal blastogenesis, the generation of multivesicular bodies (MVBs), followed by the fusion of these bodies with the plasma membrane, and finally, the release of exosomes into the extracellular space. The four protein complexes and accessory proteins that accompany the ESCRT mechanism both facilitate the storage of cargo at the endosomal membrane and cause the budding and scission of the endosome membrane containing those cargos.[22] The process of exosome formation involves

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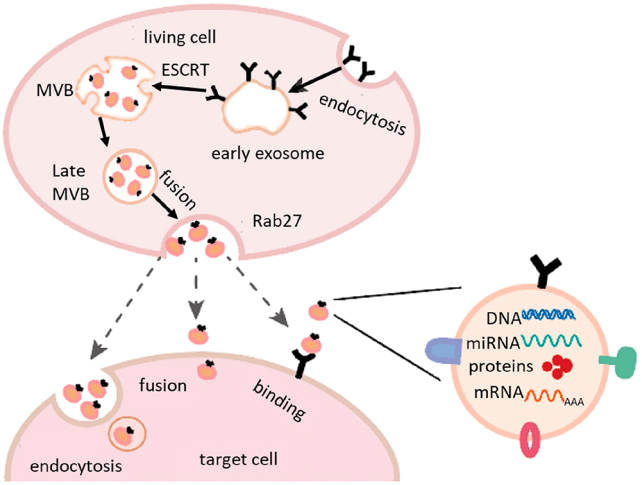

Figure 1. Schematic representation of the formation and release of exosomes. This is a finely tuned process that starts from the early endosomes in the membrane. These early endosomes are then converted into the multivesicular bodies (MVBs), which fuse with the cell membrane and are released into the extracellular space under the regulation of Rab27. Exosomes enter the target cells in three ways: fusion, endocytosis, and protein-receptor interactions, and transfer the information to the target cells. Reproduced from ref 23. Copyright 2021 Elsevier.

many vital proteins such as Ras-related protein GTPase Rab (Rab-GTPase), the tumor susceptibility gene 101 (TSG101), apoptosis-linked gene 2-interacting protein X (ALIX), syndecan-1, and sytenin-1. To convey the information to the target cells, the neighboring cells pick up these exosomes through direct fusion, endocytosis, or specific receptor binding.[23]

2.1. Composition of Exosomes

Exosomes contain numerous substances, such as specific lipids, proteins, DNA, mRNA, and noncoding RNAs, which can act as autocrine and paracrine factors.[24] Exosomes' complexity is exemplified by the transference of their contents into the cytoplasm when they move from the parent to recipient cells.[22] The complex exosomal contents are a critical determining factor of intercellular communication. They aid in transferring the characteristics from the parent to recipient cells, causing exosomes to contribute to tumor formation. Therefore, a grading system for cancer progression can be evaluated using the exosome contents.[24]

2.1.1. Exosomal Proteins. There are several groups of proteins carried by the exosomes: (i) membrane transport and fusion-related proteins such as annexin, Rab-GTPase, and the heat shock proteins (HSPs), Hsp60, Hsp70, and Hsp90; (ii) tetraspanins, also known as the four-transmembrane cross-linked proteins, such as CD9, CD63, CD81, CD82, CD106, ICAM (intercellular adhesion molecule)-1, Tspan8; (iii) MVB-related proteins, for instance, ALIX and TSG101 which act as the stereotypical biomarker for the characterization of exosomes; (iv) other proteins involved with cell adhesion-related proteins and participating cytoskeletal construction, such as integrins, actin, and myosin. All these proteins are essential components of the exosomes.[24] Similarly, TDEs secreted by the lung cancer cells have several proteins involved in tumor development, such as CD91, LRG1, Galectin-9, EGFR, and Wnt5b.[25]

2.1.2. Exosomal Nucleic Acids. Exosomes also contain nucleic acids such as mRNAs and noncoding RNAs like miRNAs, lncRNAs, circRNAs, rRNAs (rRNAs), tRNAs (tRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), and piwi-interacting RNAs (piRNAs). These RNAs can play specific functional roles and are transported by exosomes from parent cells to recipient cells.[26] In addition to RNA, studies that relate to DNA are emerging. Balaj et al.[27] and Kahlert et al.[28] found tiny fragments of single-stranded DNA and large fragments of double-stranded genomic DNA in exosomes, respectively,[22] implicating that genomic DNA mutations can be determined from exosomes. Cazzoli et al.[29] investigated the expression of plasma exosomal miRNAs in lung adenocarcinoma, pulmonary granuloma, and healthy smokers. They determined that exosomal cargo is also a significant source of micro-RNAs that could be used to differentiate lung cancer patients from healthy people.

2.1.3. Common Exosome Surface Proteins and Exosomal miRNA Biomarkers for SCLC. Surface proteins can aid in differentiating TDEs from host exosomes. However, many surface markers are shared by exosomes from different cancer cell lines and between tumor and nontumor tissues. Exosome membrane proteins play a pivotal role in exosome capture. It offers the opportunity to improve the specificity of exosome diagnosis.[30] During the biogenesis of exosomes, the collection of membrane proteins exposed on the surface of exosomes formed from cancer cell endoplasmic reticulum membranes correlates well with cancer cell membrane proteins. This finding suggests that exosomal proteins can act as tumor markers. In addition to exosomal protein, exosomal miRNAs also can be used as tumor biomarkers.[31] Mao et al.[32] showed the critical role of exosomal miRNA-375-3p in modulating vascular endothelial barrier integrity and SCLC metastasis. miRNA-375−3p has the potential for monitoring metastasis and directing clinical treatment for SCLC patients. SCLC-derived exosomes enhanced in miR-375-3p could disrupt blood barriers by targeting the vascular TJ protein claudin-1, making SCLC metastasis easier. The study by Poroyko et al.[8] demonstrated that one miRNA (i.e., hsa-miR-1180) distinguished SCLC from controls, while the other three miRNAs investigated distinguished SCLC samples before and after therapy. Contrastingly, no miRNAs for NSCLC were found to differentiate between case, control, and treated patients. Moreover, a comparison of SCLC and NSCLC samples determined that 13 miRNAs could reliably distinguish SCLC and NSCLC patients. The three miRNAs (i.e., hsa-miR-331-5p, hsa-miR-451a, and hsa-miR-363-3p) were able to determine between SCLC and NSCLC cases with 100% sensitivity and specificity, thus demonstrating miRNAs' potential role as promising candidates for differentiating NSCLC and SCLC.[8] A concise form of similar miRNAs can be found in Table 1, along with other significant exosomal proteins and micro-RNAs.

3. EXOSOME ISOLATION TECHNIQUES

The many characteristics of exosomes, including density, shape, size, and the associated surface proteins, are exploited by the techniques used to isolate exosomes in sufficient quantity and purity. These techniques include ultracentrifugation, chromatography, ultrafiltration polymer-based precipitation, and affinity capture on antibody-coupled magnetic beads.[39] These were compiled by Li et al.[40] in a convenient table for exosome isolation techniques. This section will be a

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Table 1. List of Some of the Key Exosomal Proteins and Micro-RNAs for the Detection of SCLC

Biomarker molecules	Sample	Isolation methods or major assays performed	Utility	Potential of biomarker molecules	Ref
CD9, CD151, CD81, CD171, CD63, TSPAN8	Plasma	Extracellular vesicle array	Diagnostics	When used to identify cancer patients, CD151, CD171, and TSPAN8 worked well. The collected exosomes were detected and visualized using the CD9, CD81, and CD6 antibodies.	33
TSG101, miR-665	Pleural effusion (PE) samples	High-throughput sequencing	Diagnostics and therapeutics	This approach could enable personalized immunotherapeutic regimens in patients with SCLC and other cancers. Anti-TSG101, along with other antibodies were used to extract the exomes. miR-665 is one of the most elevated exosomal miRNAs from NSCLC and SCLC.	34
S100A16	Specimens	In vitro cell coculture system	Therapeutics	Increased S100A16 has been found to actively contribute to SCLC cell survival by controlling mitochondrial activity, making S100A16 a key potential target in SCLC brain metastasis.	35
TGF-β and IL-10	Cell lines	ELISA	Therapeutics	Studies indicated that they both regulated the cellular migration of tumor cells, Therefore, if further detailed information into the contents of exosomes is undertaken, it is possible to enhance therapeutic methods with these biomarkers.	36
SAA2, APOC2, LPA, CFHR4, APOB, C1R, PRDX1, F13B, MASP1, C1QA, F13A1, HPR, OIT3, F11, FCGBP	Microvesicles and exome samples	Ultracentrifugation and label-free mass spectrometry	Diagnostics	These proteins were found to have altered expressions in SCLC compared to NSCLC and other cancers. Therefore, with further validation, these proteins could be employed as candidate markers.	7
FECR1	Serum	FLI1 shRNA knockdown and CRISPR Cas9 knockout	Diagnostics and prognosis	track disease progression of SCLC.	37
miR-375-3p	Plasma samples	qRT-PCR	Therapeutics	miR-375-3p was found to play a critical role in SCLC cells both in vitro and in vivo thus, analyzing this miRNA in patients could aid in monitoring metastasis.	32
miR-141	Plasma and serum	qRT-PCR	Therapeutics	miR-141 plays an active role in SCLC angiogenesis via the miR-141/KLF12 pathway, demonstrating its potential as a novel target for antiangiogenic therapies for SCLC patients.	38
hsa-miR-331-5p, hsa-miR-451a, hsa-miR-363-3p, hsa-miR-1180, hsa-miR-203	Serum	miRNA sequencing	Treatment	hsa-miR-331-5p, hsa-miR-451a, and hsa-miR-363-3p were able to determine between SCLC and NSCLC cases with 100% sensitivity and specificity. Exosomal miRNA was found to be easy to identify SCLC but challenging to track treatment progression.	8

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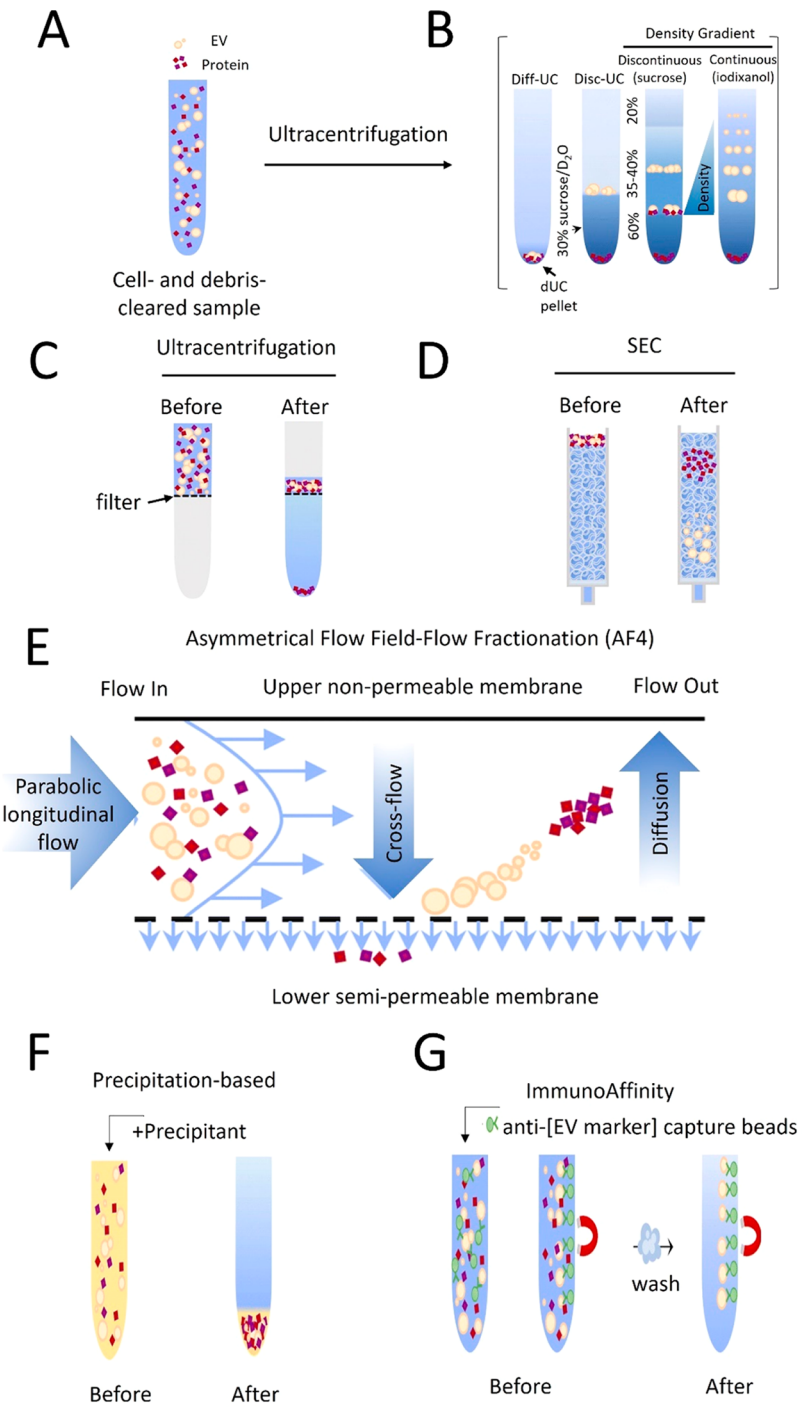

Figure 2. Graphical summary of the major EV isolation techniques. (A) The starting sample is a cell- and debris-cleared biofluid containing EVs and suspended proteins. (B) Ultracentrifugation produces an EV pellet with proteins (dUC pellet), which can be purified further using discontinuous ultracentrifugation (disc-UC), which is like floatation in a sucrose cushion, or by the density gradient (DG) ultracentrifugation, which can be done in a discontinuous gradient utilizing different sucrose solution or in a continuous, self-making gradient using iodixanol solutions (OptiPrepTM). Proteins and distinct EV populations are separated in this fashion based on their density. (C) Ultrafiltration is a dead-end filtration technology that separates molecules based on the filter pore's molecular weight cutoff (size). It creates a mixed sample of EVs and proteins while allowing for a significant reduction in the sample volume. (D) In SEC, the molecules larger than the matrix pores (EVs) elute first. At the same time, smaller particles within the fractionation range (proteins) are slowed by entering the matrix bead pores and eluting later. (E) In AF4, particles are pushed toward the semipermeable membrane by a crossflow (field) perpendicular to the longitudinal laminar flow. Particles smaller than the membrane pore are eliminated through the membrane. According to their size, retained ones migrate away due to diffusion and flow in the equilibrium position of the two forces (field and diffusion). Because the longitudinal flow's velocity increases in a parabolic pattern, smaller particles near the flow's center move faster and eluted before larger ones. Proteins and EV populations of various sizes are isolated in this manner. (F) Precipitation-based isolation concentrates all particles into a single pellet by adding water-excluding precipitants like PEG. (G) Immunoaffinity isolation is based on capturing EVs with a particular antibody that identifies an EV-specific marker, which is then attached to beads that may be separated by centrifugation or magnetic separation. Reproduced from ref 56. Copyright 2019 Springer Link.

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continuation of their work and will discuss various isolation strategies.

3.1. Ultracentrifugation

Ultracentrifugation is one of the most widely used and published techniques and is considered the gold standard for exosome isolation[41] (Figure 2A and B). It accounts for 56% of all exosome isolation procedures used by exosome and extracellular vesicle researchers.[42] Ultracentrifugation can be categorized into differential centrifugation and density gradient ultracentrifugation. Differential centrifugation separates the vesicles and other subcellular particles based on their sedimentation rate. Typically composed of multiple centrifugation steps, the technique initially subjects a lysate to low-speed centrifugation (300*g* for 10−15 min) to remove cells and apoptotic debris. Sequentially higher-speed centrifugations (20 000*g* for 30 min) of the supernatant remove larger vesicles. Typically, only three centrifugations are required to precipitate the exosomes, with final high-speed centrifugation at 100 000*g* for 2h. The bigger vesicles, which may include protein aggregates, apoptotic bodies, and other EV forms, cannot be separated from the tube because the larger particles toward the tube's top require a high-speed spin. This reduces sample purity and causes exosome contamination. A potential solution would be to resuspend and recentrifuge each pellet in a buffer solution (e.g., phosphate-buffered saline (PBS)), allowing for the removal of impurities. However, this will not allow for absolute separation.

A sucrose density gradient with a centrifugation step is one of the better alternatives. This method separates the vesicles based on their flotation densities, allowing them to float upward into an overlaid sucrose gradient. The proteins or impurities are pelleted at the bottom of the tube and easily removed, allowing for aggregate-free exosome separation.[41] Pedersen et al.[7] utilized this technique to isolate exosomes from the plasma of SCLC patients. The sample was centrifuged at 20 000*g* for 30 min at 4 °C and at 100 000*g* for 1 h at 4 °C to obtain a microvesicles and exosomes pellet. With subsequent washing and chemical treatment, the separation process aided in identifying 17 unique proteins for exosomes. Similarly, Mao et al.[32] employed ultracentrifugation further to understand the role of exosomal miRNAs in SCLC as regulators in metastatic processes. The isolated SCLC-derived exosomes were then observed through fluorescence microscopy. While utilizing an ultracentrifuge is desirable, it necessitates the purchase of expensive machinery and long periods of centrifugation, limiting its applications. Additionally the high speed of spinning, can damage the exosomes, resulting in loss of their structure and integrity. The overlapping size distribution of platelets and various membrane vesicle populations also poses a significant challenge for exosome preparation through differential ultracentrifugation.[43] Ultracentrifugation alone cannot distinguish between exosome subpopulation or other microparticles of similar density and size, such as protein aggregates, nucleic acid complexes, and lipids.[44] To overcome these limitations, ultracentrifugation in combination with sucrose gradient ultracentrifugation, or immuno-isolation is often used.[43]

3.2. Size-Based Techniques

Ultrafiltration is a size-based method for isolating exosomes (Figure 2C). This method uses membrane filters with specific molecular weight or size exclusion limits. Lobb et al.,[45] in their comparative study on different exosome isolation methods for lung cancer diagnosis, demonstrated that ultrafiltration isolated the most number of particles (<100 nm) compared to ultracentrifugation. Even though ultrafiltration could provide pure vesicles, removing contaminating proteins remains a major disadvantage. This method is faster than ultracentrifugation and does not rely on expensive instrumentations.[46] Heinemann and Vykoukal[47] developed a gentle and scalable isolation method to combat ultracentrifugation's heavy-handedness. Sequential filtration was designed as a high-throughput method ideally suited for various large-volume biofluids, such as urine, lavage fluid, and cell-conditioned media.[48] This simple method was broken up into three steps: dead-end (normal), tangential flow, and track-etched membrane filtration. Dead-end filtration removes cells, cell debris, and large EVs. Subsequently, tangential flow filtration is conducted to remove nonexosome-associated proteins, biomolecules, and small nonexosome particles while concentrating the exosomes. The final track-etched membrane filtration is used further to filtrate a size-defined division of exosomes and nonexosomal particles. Although yet to be widely accepted, this gentle approach for isolating and concentrating EVs is beneficial for its scalability and production of highly purified EVs.

Size exclusion chromatography (SEC) is another size-based separation technique used in exosome isolation and has a simple working principle (Figure 2D). It separates solutes of various molecular weights as they move through an aqueous media using a column of starch and water. Biomolecules smaller than the column's pores can pass through one column's porous stationary phase, but they move more slowly because of these pores. At the same time, the larger biomolecules cannot enter the pores due to the obstruction by smaller molecules and are washed away.[49] Jeong et al.[50] isolated lung cancer-specific exosomes using SEC. The size of isolated exosomes was verified by NTA and was found to be 30−150 nm. The isolation process aided in determining an approximately 5.8-fold increase in exosome concentration in the patients compared to the healthy controls

The most enticing characteristic of SEC in terms of exosome-based biomedical research is its ability to preserve the biological activity of the separated exosomes.[51] This is largely due to the tender separation process of employing passive gravity flow. Therefore, it does not affect the structure and integrity of the vesicles.[52] The approach's gentle nature can be improved further by employing elution buffers with physiological osmolarity and viscosity.[53] Additionally, due to the size of commercially available SEC columns, minimal sample volume, as little as 15 μL, is required.[54] Moreover, the technique is more time-efficient and less labor-intensive. The process can be accomplished in as short as 15 min using selective porous materials and buffer systems. Adaptability is another advantage of SEC. Adjusting the pore size of the applied materials allows for a defined subpopulation of vesicles.[55] Finally, compared to ultrafiltration-based separation, the contact-free method of SEC provides no or minimum sample loss and high yield.[51] Given all these advantages, it is no surprise that SEC-based exosome isolation has become prevalent for exosome-based scientific and clinical research.[53] SEC still ranks highly as an exosome isolation technique despite being notoriously difficult to scale up and demanding for high throughput exosome isolation applications.[49]

Field flow fractionation is another technique for separating EVs with minimal interaction. This technique relies on the

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particle separation in a channel when subjected to crossflow from an external gradient or "field." This crossflow may be produced by applying different energies or forces such as thermal, electrical or centrifugal. However, a tangential flow induced through a semipermeable membrane can also be undertaken asymmetrically.[56] Once the crucial experimental parameters, including crossflow velocity, membrane cutoff, and channel thickness, are adjusted, this asymmetric type of field flow fractionation conducts a high-resolution EV subpopulation separation with 10 nm precision (Figure 2E).[57] Zhang et al.[58] used the AF4 technique to identify exosomes, various nanoparticles, and EV subsets in the lung tissue samples. Their study showed that the AF4 technique could be utilized as an efficient analytical tool for isolating EVs and tackling the complexities of subpopulations of heterogeneous nanoparticles.

Size-based isolation techniques for exosomes, while useful, require greater sensitivity and specificity to effectively separate exosomes from other similarly sized microparticles found in body fluids. Additionally, these techniques have a limited sample capacity and may damage or contaminate exosomes with other cellular components. To improve separation yields and purity, several attempts have been made to combine size-based techniques with microfluidic devices.[59] For example, Liu et al.[60] used a microfluidic platform based on viscoelastic separation to efficiently isolate exosomes from extremely small volumes of cell culture media samples. This method produced good recovery rates (>80%) and achieved higher exosome separation purities (>90%) compared to other EVs.[60]

3.3. Precipitation Methods

Exosomes have also been isolated using precipitation techniques (Figure 2F), which rely mainly on polymers to precipitate exosomes. The most commonly employed polymer, polyethylene glycol (PEG), effectively improves the enrichment and yield of exosomes.[61] This approach has been reported to separate numerous biomolecules and viruses from body fluids.[62] In this technique, the samples are coincubated with a PEG solution overnight at 4 °C. Following this incubation, the exosome-containing residue can be further processed using separation procedures such as filtration and centrifugation.[45] Cazzoli et al.[29] utilized the precipitation method to isolate exosomes from lung cancer samples. They used the ExoQuick exosome precipitation solution to isolate lung cancer exosomes efficiently. Then the microRNAs were extracted from these exosomes and analyzed for their potential role as biomarkers for lung cancer.[29]

ExoQuick, Total Exosome Isolation Reagent (Invitrogen, United States), ExoPrep (HansaBioMed, Estonia), Exosome Purification Kit (Norgen Biotek, Canada), and miRCURY Exosome Isolation Kit (Exiqon, Denmark) are just a few examples of commercial exosome isolation products commonly used for increasing the efficacy and efficiency in exosome isolation processes. These kits commonly rely on multistep filtration and centrifugation procedures and differ based on their efficiency and exosome quality. Lobb et al.[45] compared various isolation techniques using tunable resistive pulse sensing and protein analysis to provide a comparative analysis to indicate the efficiency and preparation purity. They concluded that the Exo-spin is better in yielding higher levels of exosome markers than ExoQuickTM kit. However, both Exo-Spin and ExoQuick have more nonexosomal protein contamination, as shown by the ratio of exosome concentration to protein with increased exosome marker expression in qEV columns with OptiPrepTM density gradient isolation.

Due to their simplicity, speed, lack of exosome damage, and equipment-less nature, precipitation-based isolation methods are more appreciable within clinical research. However, in a comparative study of two precipitation-based methods and one column-based approach for exosome isolation from various biofluids, these methods were found to have a significant disadvantage due to the presence of various contaminants from the sample resulting from coisolation. This had downstream effects in sample analysis via mass spectrometry, proteomic analysis, and RNA tests. Adding a prefiltration step or a postprecipitation purification step with subsequent centrifugation, filtration, or gel filtering makes it possible to reduce contamination with nonexosomal contaminants.[45] In doing this, complexity is added while mitigating precipitation methods' highly sought-after equipment less appeal. However, EVs isolated by precipitation methods may be coprecipitated with lipoprotein components in the samples, as these lipoproteins tend to mimic the characteristics of the extracellular vesicles and are thus copurified with them. For example, Ludwig et al.[64] showed that small extracellular vesicle (sEV) isolated by PEG-precipitation methods can be contaminated with bovine serum albumin (BSA) from cell culture media, which could then, however, be efficiently removed by follow-up ultracentrifugation. Regardless, precipitation methods are still more appealing than other methods in clinical applications when working with biofluids due to their low requirement of sample volume and are compatible with high throughput options, which is contradictory to the golden standard method, ultracentrifugation.[63]

3.4. Immuno-affinity-Based Approaches

Large amounts of proteins are known to be present in exosome membranes. Immuno-affinity purification approaches have been employed to selectively capture certain exosomes from mixed populations of biological components such as cell cultures, tissues, and bodily fluids. This technique uses the immune interaction between exosomal surface proteins (antigens) and their specific antibodies or receptors and their corresponding ligands[40] (Figure 2G). This is convenient when proteins expressed on the surface of exosomes lack soluble counterparts and are relatively quicker, simpler, and compatible with standard laboratory equipment. Immuno-affinity methods commonly use magnetic beads covalently coated with streptavidin, which can be linked to any biotinylated capture antibody (e.g., anti-CD63, anti-CD9, and anti-CD81 antibodies) with high affinity. The specificity and yield of the exosomes isolated by the immunoaffinity-based techniques are comparable to those of the ultracentrifugation. To further improve the capture efficiency, submicron-sized magnetic particles achieved 10 to 15 times more exosome capture yield compared to the ultracentrifugation method.[42] This is attributed to the larger surface area, near-homogeneous capturing process, and magnetic beads-based immunoaffinity methods' higher capture efficiency and sensitivity than other microplate-based systems.[46] Furthermore, there are no volume limitations with these methods.[40] Immunoaffinity isolation has a significant advantage in that it can sort distinct exosome subpopulations based on their surface protein expressions, as it relies on selective antigen−antibody binding.[65] For instance, exosomes can be captured directly on the surface of a microfluidic device that has been

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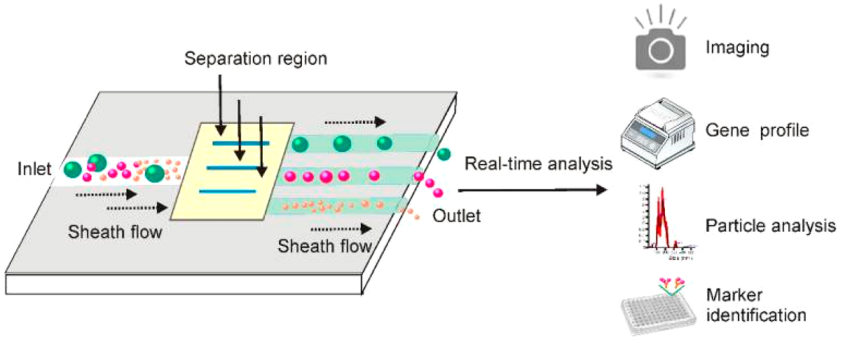

Figure 3. Exosome separation and analysis using an integrated microfluidic device. Depending on the physical and biological characteristics of the EV, multiple techniques can be used to separate the fluids' particles, including the exosomes, after adding them to the sheath medium. The microfluidic device is a prelude to various signal-detecting systems for real-time or postextraction exosome characterization to aid in in situ diagnosis. Adapted with permission under a Creative Commons Attribution License (CC-BY) from ref 53. Copyright 2020 The Authors.

functionalized with specific beads or capture agents that are coated with exosome-specific antibodies. The next step is to preincubate these antibodies with the exosome-containing serum and process the samples using a microfluidic device to determine the level of expression of particular cancer-related markers.[59]

An affinity-based isolation approach for EVs was developed by Nakai et al.[66] where it uses the T-cell immunoglobulin domain and mucin domain-containing protein 4 (Tim4) protein for capturing EVs. The extracellular domain of murine Tim4 was fused to the Fc fragment of the human IgG. The Tim4-Fc protein was immobilized on magnetic beads and used to capture EVs in the presence of Ca^{2+}. The captured EVs were then steadily released from the beads by adding a buffer that contains a Ca^{2+} chelating agent (e.g., EDTA). The yield and purity of EVs isolated using the TIM4 affinity-based approach was found to be superior to ultracentrifugation or TEI-based precipitation.[66] However, due to the presence of phosphatidylserine in many EV subpopulations, the EVs isolated by the Tim4-based approach contain exosomes and apoptotic bodies.[67]

Zhang et al.[68] developed a novel Tim4@ILI-01 immunoaffinity flake material to efficiently enrich exosomes from serum samples of lung adenocarcinoma patients. This immunoaffinity material showed an exosome capture efficiency of 85.2%, which was 5.2 times greater than the ultracentrifugation method. Similarly, Shih et al.[69] developed a magnetic bead-based method for collecting circulating extracellular vesicles for studying human lung carcinoma. They used phosphatidylserine-binding protein, annexin A5, to generate a magnetic bead-based procedure for capturing EVs from fluidic samples. Their research showed these beads, called ANX-beads, could capture up to 60% of the induced apoptotic bodies.[69]

3.5. Microfluidics-Based Isolation Techniques

The main advantage of microfluidic techniques is their ability to isolate exosomes based on their biochemical and physical properties. Microfluidic-based isolation methods are rapid and efficient and require small input sample. They also innovatively combine with other separation mechanisms such as acoustic, electrophoretic, and electromagnetic forces to extract properties of exosomal vesicles.[65] A typical microfluidic working platform is shown in Figure 3.

3.5.1. Acoustic Nanofilter. An acoustic nanofilter employs ultrasound standing waves to exert differential acoustic forces to continuously separate exosomes and other EVs based on their size and density. The particles respond to the acoustic force exerted on them based on their size and density. The larger particle requires more force to be moved causing them to migrate slower toward the pressure node. The ultrasound transducers and underlying electronics can be tuned to separate particles of any size above and below a specific desired size. Lee et al.[70] illustrated this purification procedure by extracting nanoscale (200 nm) vesicles from cell culture media and EVs in preserved red blood cell products. Additionally, they could electronically control the filtering size in real time of the experiment due to the underlying electronics and ultrasound transducers. While this methodology is still in its early development stages, its simplicity, speed, tuneability, and low sample volume allow it to be employed in the clinical setting.[65]

3.5.2. Magnetic Nanowires. Elongated magnetic nanowires (MNWs) doped with many magnetic nanoparticles (MNPs) and biotin moieties can be used to conjugate a large number of streptavidin-modified anti-CD9, anti-CD63, and anti-CD81. Lim et al.[71] used the MNWs to rapidly isolate homogeneous exosomes with high purity. These antibody cocktail-conjugated magnetic nanowires allowed for more efficient isolation and quantification of the targeted exosomes without laborious and time-consuming steps. This allowed for an approximately 3-fold greater yield than conventional exosome isolation methods. MNWs can be used in clinical applications where a highly purified population of exosomes is required to analyze embedded protein, lipid, mRNA, and miRNA. Compared to conventional methods such as Exoquick and Invitrogen exosome isolation kits, the antibody-conjugated MNWs resulted in a nearly 3-fold increase in yield. Recently, it has been employed in characterizing lung-cancer-derived circulating exosomes in patient samples[71] Because of their small lateral size, elongated structure, high surface-to-volume ratio, and strong magnetism, the MNWs-based methods can be used to isolate exosomes with high capture efficiencies and purity with potential applications in the clinical setting.

3.5.3. Exosome Total Isolation Chip (ExoTIC). ExoITC is a filtration-based EV isolation tool developed by Liu et al.[72] This tool is a user-friendly and modular chip that aids in facilitating high-yield and high-purity EV isolation from biofluids. This method passes patient samples such as plasma, urine, and lavage through a nanoporous membrane to isolate and enrich EVs. Free nucleic acids, proteins, lipids and other small fragments are washed out, while the 30−200 nm intact EVs are collected in the membrane. Subsequent character-

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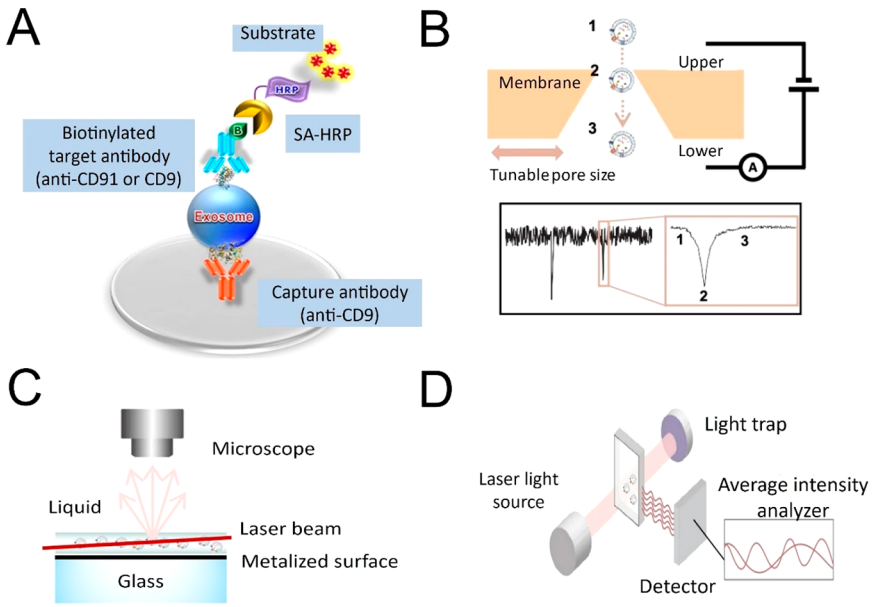

Figure 4. Examples of various conventional EVs analysis methods. (A) Schematic diagram of the sandwich ELISA for EVs detection. Adapted with permission under a Creative Commons Attribution License (CC-BY) from ref 75. Copyright 2014 The Authors. (B) Scheme and the corresponding figure for the drop in ionic current measured across the pores in tunable resistive pulse sensing. Adapted with permission under a Creative Commons Attribution License (CC-BY) from ref 91. Copyright 2022 The Authors. (C) Schematic diagram of NTA. The NTA device consists of a laser source, a glass prism to finely focus laser beam, and an optical microscope to collect light scatter from all particles in the region of interest. Adapted with permission from ref 92. Copyright 2020 Wiley. (D) The workflow of a DLS platform. Adapted with permission under a Creative Commons Attribution License (CC-BY) from ref 91. Copyright 2022 The Authors.

ization using NTA and transmission electron microscopy (TEM) demonstrated that ExoITC achieved 4−1000 times higher yield than ultracentrifugation. Moreover, the exosomes isolated via ExoTIC method show an increased expression of some miRNAs in NSCLC cell lines (HCC827 and H1650) compared to ultracentrifugation.[73]

4. DETECTION AND CHARACTERIZATION OF EXOSOMES

4.1. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is one of the most widely used techniques for detecting EVs.[74] This technique uses the sandwiching of an antibody, surface, and antigen of interest to immobilize the target. One of the significant limitations of ELISA-based exosome detection techniques is the nonspecific adsorption of the biomolecules during identifying exosomes from complex body fluids.[41] Ueda et al.[75] identified CD91 as a lung cancer adenocarcinoma-specific antigen on exosomes. They developed a sandwich ELISA with anti-CD9 coupled with highly porous monolithic silica microtips for a large-scale replication study to validate further the screening reliability of the identified exosome surface antigen CD91 (Figure 4A). The author's simplistic device has the potential for biomarker discovery and a wide range of omics studies about exosomes. In another study, Yamashita et al.[76] utilized ELISA with a capture antibody (anti-CD 81) to check the potential role of the epidermal growth factor receptor on the exosome membrane as a potential biomarker for lung cancer diagnosis. Their work demonstrated significantly higher exosomal epidermal growth factor receptor expression levels in 5/9 cancer cases compared to the standard control. Although ELISA-based platforms could be helpful in cancer diagnostics, many components of the platforms still need to be appropriately optimized, such as exploiting radioisotopes or fluorescence and affinity maturation of antibodies.

4.2. Western Blotting

Western blotting, also known as immunoblotting, is a technique that relies on the application of specific antibodies before gel electrophoresis is used to separate and visualize the proteins depending on the nature of the sample or the gel. This technique is frequently employed in EV studies to determine the presence of purified exosomes through their specific surface proteins. A mixture of proteins is sorted based on molecular weight and type through gel electrophoresis. These products are then placed in a membrane, where each protein forms a band.[49] Cao et al.[77] used this method to study the potential role of the Profilin 2 protein in promoting growth, metastasis, and angiogenesis of SCLC and confirmed the presence of exosomes. The Western blot analysis demonstrated the expression of exosome markers of Alix and TGS101. Similarly, Jin and Yu[78] employed this method to detect exosomal markers in their study on hypoxic lung cancer cell-derived exosomal miR-21. Western blot displayed the upregulation of p-PI3K and p-AKT expression within the exosomes from hypoxic lung cancer cells compared with exosomes from normoxic lung cancer cells. Due to its ease of use, broad accessibility, and ability to detect exosomal surface and internal proteins, Western blotting is a widely used exosome analysis technique. Additionally, it aids in differentiating the molecular weight of target exosome proteins in various subpopulations.[42] As a limitation, Western blot requires a more comprehensive workflow, technical handling, and expertise while needing to be more adaptable to high throughput when compared to ELISA.[49] It is also unsuitable for multiplexing, and the specificity and reproducibility are limited by the antibodies' quality.[65]

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4.3. Tunable Resistive Pulse Sensing (TRPS)

TRPS measures the particle size, concentration, and zeta potential of particles as they move through a nanopore. "Tunable" indicates that the nanopore may be adjusted in size in order to filter and detect specific particles. The "resistive pulse sensing" principle monitors the current flow through a hole, and a change in current can be read when a particle passes through the aperture (Figure 4B). The pore membrane's flexibility allows for real-time pore size optimization.[41] TRPS has been utilized to measure a wide range of nanoparticle suspensions, including magnetic beads, DNA/protein particle hybrids, and biological particles such as cyanobacteria and viruses. Several investigations have also measured the exosome particle size and concentration distributions using TRPS.[79] qNano employs this technique with a polyurethane membrane to detect the movement of particles. The pore size provides flexibility to analyze particles of a broad size range (40 nm to 10 μm). Moreover, qNano relies on a limited number of consumables and does not require carrier gases, fluidics, or optics.[80] For example, Feng et al.[18] used qNano to measure the sizes of lung adenocarcinoma-derived exosomes. The exosome pellets were mixed with the 500 μL of sterile phosphate-buffered saline, filtered through a 0.22-μm syringe filter, and finally analyzed by NanoSight. The qNano and Nanosight analysis was compared with TEM and Western blot identification of CD63 and CD9 and found the vesicles were all within the range of 40−105 nm. Niu et al.[81] recently utilized this method to measure the size and distribution of exosomes. The qNano analysis revealed that the vesicles were approximately 80 nm in diameter. One significant limitation of the TRPS method is that it does not provide any information about the origin of exosomes.[41]

4.4. Nanoparticle Tracking Analysis (NTA)

Nanoparticle tracking analysis has found its way to being the most popular method characterizing the size and concentration of exosomes. This method relates the particle size to the rate of Brownian motion to determine the size distribution profile of nano and microparticles suspended in liquid solution (Figure 4C). A laser beam interacts with the exosome nanovesicles in NTA, while a charge-coupled device camera captures the scattered light. A comparison of NTA with flow cytometry using human placental exosomes shows that NTA can measure as small as ≈50 nm biological nanovesicles with excellent sensitivity.[82] When compared to electron microscopy and atomic force microscopy, NTA has the potential to characterize nanovesicles in a large.[49] Fan et al.[83] used NTA to study exosomal markers for the specific early diagnosis of lung cancer. The average size of the exosomes was determined as 120 ± 80 nm within expectation, and the concentration was 1.5×10^7 particles/mL. The findings revealed that various types of exosomal markers expressed themselves at different levels. Revealing information on a potential substitute for measuring exosomal markers in certain diseases using clinical bioassays, Zhao et al.[84] used NTA as a comparison against the CRISPR method to determine the concentrations and size of exosomes extracted from lung cancer cell lines. The concentration of exosomes was 6×10^7, 6×10^6, 6×10^5, 6×10^4, 3×10^3, and zero particles per microliter. This demonstrates the efficacy of the NTA method for utility as a benchmark to compare with other methods.

The sample preparation for NTA analysis is straightforward and rapid. Additionally, the samples can be recovered in their native state after measurement. Despite its reliability in fundamental research, NTA has significant limitations in characterizing exosomes in clinical samples.[85] These come from the time-consuming procedures involved in data acquisition. Unlike flow cytometry, which can analyze 1000 particles in less than a second, NTA takes approximately 10 min. Also, long analysis times cause the bleaching of the fluorescent dye (i.e., exosomes are stained with common fluorescent dyes, such as green fluorescent protein or antibodies conjugated with the fluorescein isothiocyanate). Furthermore, this tool cannot analyze the biochemical composition of distinct subpopulations of exosomes.[41]

4.5. Dynamic Light Scattering (DLS)

Like NTA, dynamic light scattering (DLS) exploits the characteristics of Brownian motion to determine particle tracking. This technique determines the size distribution profile of particles with several micrometers in diameter (Figure 4D). DLS is frequently used to validate exosome subpopulations' sampling by measuring EV size distributions.[49] By monitoring the variations in scattered light intensity and then using a mathematical model based on Brownian motion and light scattering theory, it is possible to calculate the size distribution of these EVs. The mean signal amplitude of extracellular vesicles depends on their concentration, diameter, and refractive index.[46] By using DLS, we can expect accurate size distributions of the monodisperse samples (samples containing one specific size of extracellular vesicles). However, the size distribution of polydisperse samples such as human plasma is not precise due to a broad range of particles in the sample altogether with inaccurate and outdated weighting algorithm for analysis.. Generally, DLS requires prerequisite knowledge of the shape of the size distribution in order to acquire accurate results.[49]. In their study of human lung epithelial adenocarcinoma cancer cells (A549), Gurunthan et al.[86] utilized DLS to measure the size of exosomes and the platinum nanoparticles (PtNPs). This, along with other characterization techniques such as NTA and TEM, proved that PtNPs can potentially increase exosome production in A549 cells.

4.6. Flow Cytometry

Flow cytometry, a physical form of analysis, is used to observe EVs visually. However, for the EVs to be analyzed, flow cytometry requires prior knowledge of the EV's protein composition. Furthermore, it requires a single particle suspension, which can be challenging to achieve when the EV concentrations are high or if they aggregate during the isolation procedure.[12] Multiple particles are observed simultaneously when EVs aggregate, resulting in incorrect data. For flow cytometry analysis, EVs must be immobilized on the surface of beads (either by immunocapture or covalent attachment). The EVs are then subjected to a fluorescently conjugated antibody against an antigen known to be or is anticipated to be expressed on the surface of the EVs after immobilization. An epifluorescent microscope (EPI) can be used to visualize the EV, bead and fluorescent antibody coupling prior to flow cytometry analysis. The sample then creates a fluorescent signal through the laser of the flow cytometer.[87] This method allows for high-throughput EV analysis and EV quantification and classification based on antigen expression. Rim and Kim[88] used this technique to perform a quantitative analysis of exosomes derived from murine lung cancer cells and classified the cancer-specific

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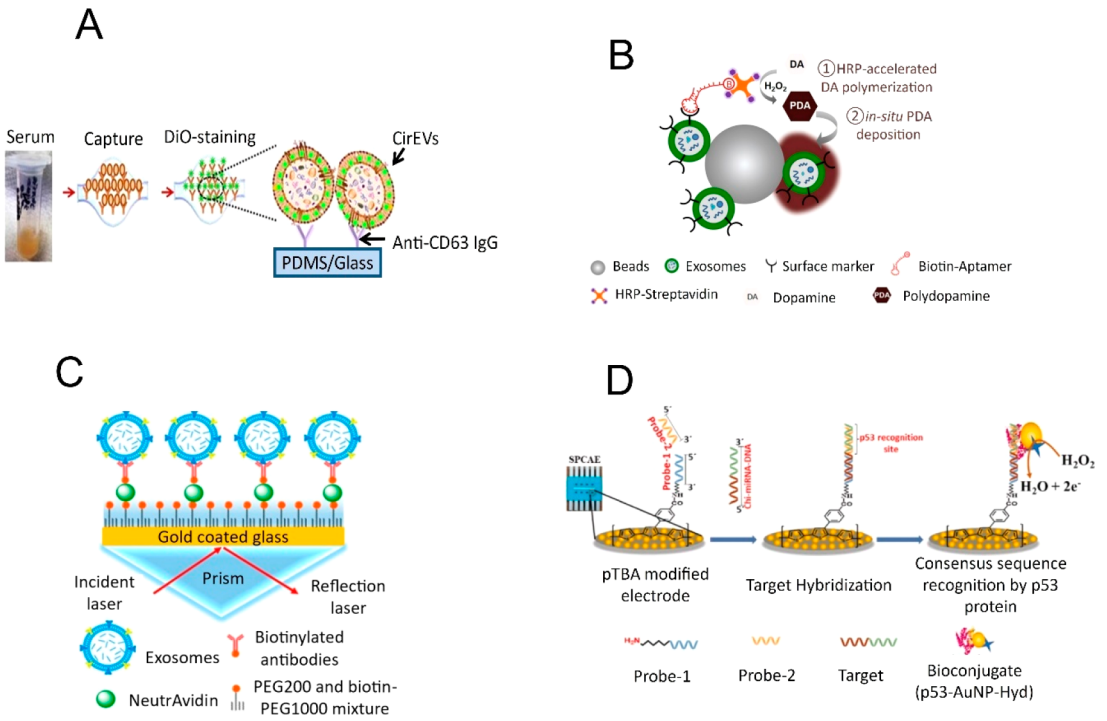

Figure 5. Examples of microfluidics-based exosome detection methods. (A) ExoChip's technique for exosome immobilization and characterization. Reproduced from ref 93. Copyright 2014 Royal Society of Chemistry. (B) ExoAptaSenosr schematic representation of an exosome detection HRP accelerated aptasensor for dopamine polymerization and deposition. Reproduced from ref 96. Copyright 2020 Elsevier. (C) Schematic illustration of the SPR-based detection of exosomes. Reproduced from ref 100. Copyright 2018 American Chemical Society. (D) Electrochemical detection of target Chi-miRNA-DNA on a screen-printed carbon array electrode. Reproduced from ref 107. Copyright 2022 Elsevier.

proteins and miRNA as diagnostic markers. Gao et al.[89] employed the rolling circle amplification (RCA)-assisted flow cytometry approach for examine protein patterns in exosomes from various lung cancer cell lines. The combination of amplification and flow cytometry allowed an extremely low detection limit of 1.3×10^5 exosome/mL. They also reported an enhanced expression of MUC1 and PD-L1 exosomal surface markers in lung cancer patient samples compared to healthy individuals.

Fluorescence-activated cell sorting (FACS) is another type of flow cytometry that allows for sorting exosome nanovesicles using fluorescent labeling.[90] Exosomes can be captured and sorted based on targeted surface protein expression using specific antibodies labeled with fluorescent dyes. FACS provides information about each cell by detecting the fluorescence emitted from the flowing samples. As cells move across the detecting region, they can instantly detect their fluid state and simultaneously assess their size, function, and intracellular composition. Additionally, FACS can only separate a specific type of cell.[88] FACS has been used to characterize exosome subpopulations in recent years.[49] Rim and Kim[88] developed a FACS-based technique for analyzing the murine lung cancer cell exosomes. The exosomes were initially isolated using CD9- or CD63-coated antibodies. FACS was used to analyze exosomes after staining the sample with an exofluorescein isothiocyanate exosome staining solution. According to their study, LA-4 lung cancer cells had an upregulated amount of CD63-specific exosomes.

4.7. Microfluidic-Based Detection Techniques

The following detection techniques are coupled with microfluidic-based sample processing steps.

4.7.1. Optical Detection. *4.7.1.1. Fluorescence.* Because of its high sensitivity and good accuracy, fluorescence imaging has been widely used in microfluidics-based exosome analysis devices.[92] Kanwar et al.[93] developed an integrated microfluidic platform (Figure 5A), ExoChip, to simultaneously capture and quantify exosomes directly from blood serum. The chip uses antibodies against CD63-based capture, and a fluorescent carbocyanine dye (DiO) based quantification. The ExoChip follows a three-step procedure: (i) A serum sample from a healthy or diseased individual is inserted into the inlet, precoated with anti-CD63. CD63's abundance on exosomes provides selectivity to the isolation procedure. (ii) The captured exosomes were then stained with fluorescent carbocyanine dye (DiO), which specifically stains the membrane vesicles immobilized in the chip. This allows the visualization of microscopically invisible small vesicles (30–300 nm) for imaging purposes. (iii) Fluorescently stained vesicles were determined on-chip using a plate reader. In another study, Kang et al.[94] utilized the microfluidic device Exo-Chip integrated with phosphatidylserine (PS) specific protein to isolate exosomes from the lung cancer cell line A549. Their results demonstrated that the device isolated 35% more A549-derived exosomes than an antiCD63-conjugated device and achieved 90% capture efficiency for cancer cell exosomes compared to 38% for healthy exosomes.

4.7.1.2. Colorimetric Detection. The integration of colorimetric technologies into microfluidic platforms for detecting exosomes is ideal for point-of-care testing due to their easy operation and simple signal readout.[95] Xu et al.[96] developed a colorimetric ExoAptaSensor (Figure 5B) for detecting cancer-derived exosomes, including lung cancer derived-exosomes from the A549 cell line. The sensor

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employed aldehyde latex microbeads to anchor exosomes through aldimine condensation. Using the streptavidin−biotin affinity interactions, CD63-specific biotinylated-aptamer and streptavidin-conjugated HRP were added, followed by the rapid addition of freshly prepared dopamine (DA) solution. HRP accelerated the colorimetric reaction, forming a polydopamine (PDA) which is a colored product. Due to PDA's excellent reactivity to the amine, sulfhydryl, and phenol groups of exosomal surface proteins causes increased binding of the PDA product to the target exosome.[97−99] The generated color in the substrate solution can be quantified using absorbance measurement (quantitatively) or visualization with the naked eye (semiquantitatively or qualitatively). These absorbance results directly correlate to the expression level of the CD63 marker on the exosomes. Moreover, Xu et al.[96] demonstrated the detection of exosomal biomarkers such as HER2 expression for the diagnosis of breast cancer cell, HCC1954. An accurate colorimetric quantification of HER2 from derived cell cultures with a detection limit as low as 7.77×10^3 particles/mL, 3−5 orders of magnitude better than conventional dot-blot methods was obtained.

4.7.1.3. Surface Plasmon Resonance (SPR) Detection. A label-free optical detection method called surface plasmon resonance (SPR) imaging monitors and analyses biomolecular interactions in real-time. SPR is a promising method for characterizing exosome subpopulations with high detection specificity and sensitivity.[49,96]. In surface plasmon resonance (SPR), when polarized light impacts the interface of two materials having different refractive indices at a critical angle, it can produce the resonance of the free electrons in the metal layer. Therefore, substantially reducing the reflected light at that specific angle. This reflected light can entirely vanish at a certain angle, known as the SPR angle. Dynamic SPR angle change can be observed, resulting from the binding interaction between biological molecules.[44] Integrating with microfluidics, SPR becomes more cost-effective and offers rapid detection. Therefore, microfluidic-based SPR approaches gradually take the lead in exosome detection.[92] Liu et al.[100] used exosomal epidermal growth factor receptor (EGFR) and programmed death-ligand 1 (PD-L1) as biomarkers to demonstrate the feasibility of a compact SPR chip in lung cancer diagnosis (Figure 5C). The chip's design consisted of a 2 nm titanium film deposited on a glass slide, followed by a 49 nm gold film used as an SPR chip. The gold film was used as the refractive layer, while the titanium film improved the gold film adhesion and therefore increased the biochip's reliability. The sample wells (diameter of 6 mm) were then created by attaching a PDMS layer with a hole in the middle to the glass slide. Users could use a pipet to add/remove samples into/from the sample wells with this design. As such, mitigating additional training or the use of equipment makes it compatible with standard clinical sample-handling processes.[100] A549 lung cancer cell line cells were identified to have a higher level of exosomal EGFR than BEAS-2B normal cells. The compact SPR chip outperformed ELISA in detection sensitivity and had a similar sensing accuracy. In another study, Thakur et al.[101] demonstrated the localized surface plasmon resonance biosensor (LSPR) for the detection of the multivesicular vesicle (MV) in serum and urine samples from a lung cancer mouse model. LSPR is like SPR but is typically employed for nanoscale sensing applications as it is less sensitive to the interference. The results produced significant foresight into the membrane properties of tumor-derived exosomes and MVs. In this regard, LSPR biosensors can potentially be used for the direct detection and isolation of heterogeneous EVs.

There are some drawbacks of SPR-based exosome biosensors. Most SPR biosensors are suitable for quantifying exosomes isolated via conventional methodologies such as ultracentrifugation. Another significant limitation of SPR biosensors is their inability to support multiplex analysis.[96] Since SPR is a mass-sensitive technique, the high molecular weight targets usually result in good detection sensitivity. In contrast, the low molecular weight compounds (i.e., smaller nanovesicles) are more challenging to be detected. In addition, the SPR technique is well-known for generating false positive responses due to mass increases via nonspecific adsorption of unwanted biomolecules in the samples. Finally, in most cases, the effective working area of the SPR chip is relatively smaller, which limits its capacity for large-scale target binding and characterization of exosomes.[49]

4.7.1.4. sEV Subpopulation Characterization Platform (ESCP). Wang et al.[102] developed a sensitive, high-throughput platform known as the small extracellular vesicle (sEV) subpopulation characterization platform (ESCP) for sEV subpopulations characterization in various types of cancer, including lung cancer. The ESCP device integrated circulating nanoscopic flow with the surface-enhanced Raman spectroscopy or scattering (SERS) barcoding on a single microfluidic device to allow the ultrasensitive and multiplexed detection of the phenotypes of sEV subpopulation because each sEV carried a small quantity of the biological cargo. ESCP allows the capture of target sEVs via the on-device immunoaffinity principle. Then the captured sEVs are in situ labeled with SERS barcodes that have been functionalized with antibodies (i.e., the conjugated gold nanoparticles with Raman reporters). By utilizing the circulating nanoscopic flow within the ESCP microarrays further enhanced the assay sensitivity to detect as low as 10^3 sEVs/mL. However, this number may vary depending on the antibody affinity and the expression of biomarkers on sEVs. This highly efficient ESCP platform can be utilized to identify sEV subpopulations and could play a vital role in diagnosing cancer and monitoring its treatment.[102]

4.7.1.5. Electrochemical Detection. Electrochemical detection exploits the electrical current generated from redox reactions in the testing compound. Typically for exosomes, an antibody will bind to a selectively recognized antigen on the surface. An electroactive signal transducer generates a measurable electrochemical signal to quantify the exosome amount.[103,104] This makes electrochemical detection well suited for biomolecular analysis because of its inherent advantages, such as high sensitivity and specificity, compatibility with miniaturization, simplicity, and a relatively low detection cost. The detection is then read by various voltammetry techniques, amperometry, and impedimetric techniques.[41] Mahmudunnabi et al.[107] developed an array sensor to detect exosomal miRNAs using a conductive polymer covalently bound to the sensor probe materials for lung cancer diagnosis (Figure 5D). The sensor array magnetically isolated the specific miRNA from lysed exosomes, with a subsequent chi-miRNA-DNA formation using T4 DNA polymerase. The sensor's dual specificity echoed the attomolar level detection limit with an excellent dynamic range. Additionally, this sensor showed practical applicability for detecting four different exo-miRNAs from cell culture media and serum samples from lung cancer patients down to the femtomolar level. The developed method is reliable, requires less fabrication time, and has the

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potential to be utilized in clinical settings. In another study, Zhang et al.[105] developed electrochemical microaptasensors for successful exosome detection in serum samples of lung cancer patients, showing that their method has great potential for early cancer diagnosis. Using the micropatterned electrodes and hybridization chain reaction (HCR) dual-amplification strategy, the microaptasensors achieved a linear detection response for a broad range of exosome concentrations with a low detection limit of 5×10^2 exosomes/mL. In their study on lung cancer cells, Ahmed et al.[106] utilized inexpensive and single-use gold (Au) screen-printed electrodes (SPEs). They successfully detected the aberrantly phosphorylated EGFR and ERK protein isoforms derived from the lung cancer cell exosomes. The sensitivity was down to just 15 ng/L in samples with up to 90% excess of their nonphosphorylated (wild-type) forms. They further demonstrated the application of this platform for tracking the effects of Tyrosine Kinase Inhibitors over a period. This noninvasive method has the potential to provide new opportunities for the diagnosis of cancer and time-point monitoring of the therapeutic responses.

Often electrochemical biosensor development involves complicated fabrication steps, so the bioconjugation process must be carefully controlled to ensure assay reproducibility. Signal amplification tags and biomarkers must be carefully considered to avoid nonspecific adsorption issues in electrochemical immunoassays. Nevertheless, the combination of electrochemical approaches and microfluidic platforms can result in an efficient clinical diagnostic tool, particularly in point-of-care devices for many disease detection applications utilizing exosomal biomarkers.[41]

5. CURRENT CHALLENGES IN EXOSOME ANALYSIS AND POSSIBLE SOLUTIONS

A significant obstacle to the therapeutic use of exosomes is the need for more reliable and accurate methods to recognize and detect an enriched population of exosomes amid the other nonspecific exosomes and EVs in circulation. Given the growing interest in exosome research, there is an urgent need for an effective and reliable tool for isolating specific exosomes. However, technological limitations related to the currently accessible isolation and detection methods make exact exosome separation problematic. Furthermore, various biological obstacles must be considered to create a valid method for exosome analysis.[41]

5.1. Technical Challenges

The International Society for Extracellular Vesicles is an international scientific organization that studies extracellular vesicles. They have started recommending a standardized and evidence-based approach to analyzing extracellular vesicles.[108] This is caused by the variability in several preanalytical processes involved in exosome isolation and characterization that has affected the outcomes of exosome analysis. One of the standards and critical challenges in the sample collection method is the presence of contaminates from the activated platelet-derived vesicles due to the physical forces associated with the blood pull. Therefore, to prevent shear stress, uniformity of the sampling sites, right-sized needles, and proper blood drawing techniques are recommended.[109] In addition, the exosome abundance often varies due to the availability and types of biofluids.[110]

Despite substantial advances in the separation and purification of exosomes, developing larger-scale batch exosome production remains a significant problem. This limits the scope of exosome-based biological studies and treatments. As a result, a simple, reproducible, repeatable, and good manufacturing practice (GMP)-compliant production platform is required. In addition, for large-scale production of the modified exosomes, additional development of GMP protocols, more automated and digital production methods, and strict quality control systems are required.[111]

Another challenge in exosome analysis is the discrepancy in the results caused by improper storage conditions (such as freezing). Samples for large-sample analyses are frequently collected from remote areas and freeze-stored before being analyzed. As it could impact quantification, it is always recommended to use freshly collected samples when conducting exosome analyses.[41]

The process of isolating and detecting an enriched subpopulation of nanosized exosomes (such as tumor-derived exosomes) among the other normal exosomes is quite challenging due to the need for consistent and specific methods. Therefore, a comprehensive strategy for precisely separating different exosome subpopulations based on biophysical and biochemical characteristics is urgently required.[49]

Exosomes have been separated throughout the past few years using traditional exosome isolation procedures according to their biophysical or biochemical characteristics. For example, differential ultracentrifugation, one of the most popular techniques for size-based exosome isolation, ignores the immune profiles of different exosome subpopulations. Similarly, exosomes are frequently lost in ultracentrifugation, and copelleted impurities occur during the isolation process. On the other hand, despite having higher immune selectivity, immunoaffinity-based isolation techniques are limited due to the lower yields of isolated exosomes and costly antibodies. Therefore, it has been proposed to combine size-based and immunoaffinity-based methods into an integrated approach to utilize the advantages of various isolation methods to accurately separate the distinct exosome subpopulations.[41]

It has been demonstrated that clinical-grade exosomes can be produced by combining ultrafiltration and ultracentrifugation methods. Similarly, ultracentrifugation can first be utilized for concentrating large volumes of samples and thoroughly processing the bulk exosomes before being incubated with antibodies or aptamers-coated superparamagnetic nanoparticles and further separating the exosomes by immunoaffinity.[112] Microfluidics can provide a miniaturized platform for integrating feasible ultrafiltration and magnetic isolation techniques. In addition, multiplex exosome surface proteins might be used simultaneously to separate distinct subpopulations effectively. However, the issue with microfluidics technologies is that all the microfluidic devices use the same exosome sorting method, thus resulting in limited yield or specificity. Moreover, their low processing capacity may hinder the downstream analysis due to insufficient nucleic acids and proteins in the isolated exosomes.[112]

5.2. Biological Challenges

As exosomes still have unknown characteristics, several genetic, physiological, and environmental factors linked to sample heterogeneity can influence exosome isolation and analysis. For example, disease-specific exosomes vary between people depending on various factors such as gender, age, gender, body mass index (BMI), immunity, and being found in healthy

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people.[113] Therefore, choosing the best-matched control for a sizable cohort of heterogeneous samples is complicated. Hence, more systemic research is required to understand the effects of sample heterogeneity on exosome biogenesis, functionality, and quantity. It is imperative to establish a predesigned sample control bank that contains controls from all potential variants of the target population, such as different ages, sexes, races, and physiological states.[41] There are just a few documented methods for effectively identifying disease-specific exosomes in the backdrop of normal exosomes, despite recent developments that have improved the efficacy of separating exosomes from other extracellular vesicles.[114,115] Exosomal cargo is protected from harsh conditions within the exosome's encapsulated protective environment; for example, the exosomal miRNA is protected from the ribonuclease (RNase) mediated destruction of RNA. However, this significant advantage of the exosomal miRNA might create a major challenge for analyzing miRNA because it must be released from the isolated exosomes, which incurs numerous additional complex steps in the analysis.[116] Several fundamental questions about exosome functionality and content are yet to be answered.[117] For instance, it is still being determined whether the transport of exosomes and their uptake by distant recipient cells are due to phagocytosis or uptake by specific receptors of the distant recipient cells.[118] The challenge for developing exosome biomarkers is the need for large-scale studies to demonstrate that the exosome liquid biopsy could be a suitable alternative to the tumor tissue biopsy. Even though exosome-mediated therapies, diagnostics, and prognosis appear promising, additional research is needed before exosomes are used in clinical applications.[110]

6. CURRENT BARRIERS AND CHALLENGES IN TRANSLATIONAL SCLC RESEARCH

The development of effective therapy for SCLC faces several challenges. Obtaining sufficient tumor tissue for molecular diagnostic studies is difficult because few patients undergo surgery, and most diagnoses are based on small biopsies or cytological samples Furthermore, due to the aggressiveness of the disease and the comorbid conditions linked with smoking, individuals with SCLC are frequently debilitated upon diagnosis and recurrence. Efforts to enhance the clinical outcome of SCLC patients will have to overcome several challenges. One recent study, for example, implies that molecular identification of circulating tumor cells could eliminate the need for more invasive biopsies.[119] Other obstacles in the SCLC research include (1) the molecular complexity of SCLC, (2) a lack of understanding of the causes of chemotherapy resistance in recurrent disease (including distinct molecular modifications acquired after initial treatment), and (3) the disease's rapid progression and high metastatic potential along with SCLC being immunologically cold therefore limits the disease response to immune checkpoint inhibitors. Furthermore, SCLC research has received little funding in recent years (possibly due to some limitations in contrast to NSCLC research tools, including an abundance of tissue and model systems). For example, in 2012, the National Cancer Institute (NCI) research portfolio had 745 lung cancer research projects, with only17 (about 2%) of those focused on SCLC.[120] However, recently the United States Congress listed SCLC in the Recalcitrant Cancer Research Act (2013), opening up funding sources outside the National Institutes of Health (NIH). Additionally, a resurgence in SCLC research (basic and clinical) has occurred, which was commenced by the genomic sequencing efforts published by George et al.[121] There have been recent breakthroughs and current research efforts to understand disease subgroups, new treatment options, new preclinical models, tumor heterogeneity and cell of origin. However, detection and effective treatment remain urgent unmet clinical needs.

The current experimental setup for SCLC research is based on the association of in vitro phenotypes with metastasis, in vivo metastasis originating from xenograft transplants, and the metastasis formed in genetically modified mouse models. Although these models provide information, each of them has limitations. For instance, the cell line-based metastatic SCLC models can depict the phenotypes acquired during the propagation in the culture.[122]

7. CONCLUSIONS AND FUTURE PROSPECTS

This Review has summarized the epidemiology of lung and small-cell lung cancer and discussed the proposed biomarkers and exosomal biomarkers in SCLC research. Additionally, we have thoroughly discussed the recent advances in exosome isolation and detection techniques. Finally, we have also addressed these techniques' significant technical and biological challenges and the major challenges of diagnosis and disease monitoring faced by SCLC research.

Despite progress in understanding SCLC, many gaps have become the subject of recent research. These include identifying lung cancer risk, high metastatic behavior in early stages and prognosis reports.[123] Liquid biopsy has just become a reality in lung cancer research and clinical practice. In addition, various ongoing research efforts have focused on exosomal cargo and its functions in the genesis and progression of lung cancer as well as their application as diagnostic, prognostic, and predictive biomarkers.[124] Proteins on the cell surface and within exosomes could be exploited as lung cancer biomarkers.[30] Several protein detection or exosome capture approaches potentially allow for cancer-type differentiation using various biofluids such as CSF, plasma, serum, saliva, urine, and ascites.[36] Exosomal miRNAs have the potential in SCLC research, and basic studies have demonstrated breakthroughs in the involvement of exosomal miRNAs and lncRNAs in SCLC.[125] However, the utilization of liquid biopsy-based methods, such as circulating tumor DNA and exosomal biomarkers for SCLC diagnosis, prognosis, and treatment selection, is still in the early stages.

Recent advancements in exosome and exosomal biomarker studies suggest that exosomes have unique features that make them an ideal tool for liquid biopsy in cancer research. A perfect cancer biomarker has the potential to demonstrate the presence of a tumor mass and its molecular characteristics in the very early stages. Since exosomes are detectable in almost every biofluid, researchers can choose a specific biofluid to identify patient exosomes based on the type of cancer. Exosomes contain DNA, RNAs, and proteins that could provide real-time information about the biological and clinical characteristics of the tumor mass.[126] Another advantage is that exosomes are highly stable due to their lipid bilayer encapsulation, which is critical because the genetic contents of the exosomal cargo reflect the parent tumor cell.[127] Additionally, exosome identification is straightforward[128] and exosomal surface proteins can play a critical role as diagnostic biomarkers in identifying various types of cancer.[88] Thus,

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exosomes are expected to be crucial biological components in liquid biopsy for both prognostic and diagnostic purposes.

The fundamental issue with SCLC is that there needs to be an efficient system for the early diagnosis and detection of SCLC. Since it is very metastatic, when patients realize the symptoms of SCLC, the cancer is already in stage 3 or 4. Therefore, there is a need for earlier, noninvasive, or minimally invasive diagnostics and strategies for the early detection of SCLC. It is now well-known that liquid biopsy-based tools offer a comprehensive approach for early detection of many diseases. Therefore, with the hope that the development of the aforementioned tools and the discovery of novel exosomes and exosomal biomarkers will improve the survival outcome of SCLC patients, we anticipate conducting several of these studies in the future.

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Notes

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