

Harnessing the potential of small extracellular vesicle biomarkers for cancer diagnosis and prognosis with advanced analytical technologies

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ABSTRACT

Small extracellular vesicles (sEVs), also referred as exosomes, have emerged as valuable indicators of cancer progression and response to treatment. They offer prospective targets for therapeutic interventions as well as insightful information about the fundamental mechanisms underlying the development of cancer. sEVs have garnered significant attention as a useful tool for liquid biopsies used in non-invasive cancer diagnosis. We discussed their potential in predicting treatment outcomes, monitoring disease progression, and classifying cancer stages and subtypes. sEVs can also shed light on how resistance to several cancer treatments, such as drug resistance, radiation resistance, chemotherapy resistance, and immunotherapy resistance develops. sEV-based cancer diagnostics have initiated clinical trials, underscoring their potential clinical value. Additionally, significant progress has been made in the development of techniques for isolating and enriching sEVs, enabling the sensitive and efficient detection of sEV proteins and nucleic acids. These advancements have resulted in enhanced sensitivity and specificity, facilitating the identification of biomarkers with low expression levels. In conclusion, sEV biomarkers offer significant potential for the diagnosis and monitoring of cancer. The utilization of sEVs in liquid biopsies presents a non-invasive method for acquiring tumour-specific information. Ongoing research and advancements in sEV-based diagnostics and therapeutics are crucial for unlocking the complete potential of sEV biomarkers in clinical settings.

Key words: small extracellular vesicle biomarkers, liquid biopsies, small extracellular vesicle-based diagnosis, small extracellular vesicle isolation

INTRODUCTION

Small extracellular vesicles (sEVs) released by diverse cell types have emerged as promising biomarkers for diagnosis and monitoring of cancer. These EVs promote intercellular communication by transferring bioactive molecules, such as proteins, nucleic acids, and lipids.^[1] A comprehensive understanding of the biogenesis of EVs is essential to comprehend their functional significance and unlock their potential as diagnostic tools in cancer.

EVs are classified into different subtypes based on their biogenesis pathways, with sEVs being the most extensively

studied subtype.^[2] sEVs originate from the endosomal pathway and are formed through a complex series of intracellular processes. Biogenesis of sEVs begins with the inward budding of the plasma membrane, leading to the formation of early endosomes. These early endosomes mature into multivesicular bodies (MVBs) through the inward budding of their limiting membrane.^[3] Subsequently, MVBs can either fuse with lysosomes for degradation or undergo exocytosis, releasing their intraluminal vesicles as sEVs into the extracellular space.^[4]

The cargo carried by sEVs is highly diverse and reflects the molecular composition of the parent cells.^[5] This cargo encompasses

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various proteins, such as membrane transporters, signalling molecules, and receptors, as along with different types of nucleic acids, such as messenger RNA (mRNA), microRNA (miRNA), and long non-coding RNA (lncRNA).^[6-8] The selective packaging of these molecules into sEVs suggests a tightly regulated sorting process during their biogenesis.^[9,10]

The ability of sEVs to transfer their cargo to recipient cells has led to growing interest in their role in cancer progression and therapeutic resistance. sEVs derived from cancer cells can modulate the tumour microenvironment,^[11] promote angiogenesis,^[12] facilitate metastasis,^[13] and contribute to immune evasion.^[14] Moreover, the specific cargo carried by cancer cell-derived sEVs can reflect the molecular characteristics of the tumour,^[15] providing valuable information for cancer diagnosis and monitoring.

Before the 1970s, surgery stood as the primary rational cancer treatment, making a significant milestone in oncology. This era laid the groundwork for the subsequent development of radiation therapy and anticancer chemotherapy. The period spanning from the 1970s to 2023 witnessed a remarkable expansion in cancer therapeutics, with the introduction of therapies such as immune checkpoint inhibitors, pharmacological hormone therapies, and chimeric antigen receptor T cell therapy. These advanced treatments enhanced the efficacy of cancer management. These therapies are positioned to retain their essential roles in cancer treatment moving forward. Furthermore, advancements in early detection methodologies hold promise in revolutionizing patient care by enabling interventions at earlier disease stages, potentially intercepting metastatic progression.^[16]

In this review, we delve into the potential of sEV biomarkers in various aspects and also discuss the utility of sEVs in liquid biopsy for predicting treatment outcomes, monitoring disease progression, and identifying resistance mechanisms. Additionally, we highlight recent advancements in sEVs isolation and enrichment techniques, as well as novel methods for detecting sEV proteins and nucleic acids. By integrating sEV biomarkers into clinical practice, we envision a transformative impact on cancer diagnostics and an overall enhancement of patient care.

Liquid biopsies offer a convenient alternative to tissue biopsies,^[17] enabling multiple sampling possible throughout a patient's treatment. These liquid biopsies encompass various biomarkers, including circulating tumour cells,^[18] cell-free DNA^[19] and sEVs.^[20] Over the past decade, circulating tumour DNA (ctDNA) was one of the most important components of liquid biopsy techniques. However, a significant challenge lies in the small fraction of ctDNA amidst the abundant background of normal

cfDNA.^[21] Among these biomarkers, sEVs stand out as particularly useful due to their higher abundance in circulation and increased stability, making them a good candidate for liquid biopsy applications. The distinct biological origins of cfDNA and EVs introduce difference in the representation of tumour heterogeneity, allowing differentiation between EV-DNA and cfDNA.^[22] The membranous structures of sEVs provide a protective shield,^[23] preventing the enclosed molecules from physical degradation. sEVs derived from cancer cells have demonstrated significant potential as biomarkers for detecting and monitoring diseases since they can be found in a variety of bodily fluids, such as blood, mucus, urine, and bronchial fluid, and sEVs derived from different cancer types carry a unique set of biomarkers (Figure 1). The non-invasive nature of acquiring cancer-derived sEVs enables sequential sampling of patients, offering valuable insights for early diagnosis, identifying cancer recurrence, assessing drug response, and stratifying patients across a wide range of cancer types. Advances in nanotechnology-driven biosensors has ushered in a new era of diagnostics, characterized by enhanced capabilities including high throughput analysis, minimal sample requirements, and cost-efficient detection of sEV biomarkers. The ongoing refinement and innovation of biosensor technologies may facilitate integration of sEV detection into clinical environments.^[24] sEV detection has the potential to greatly assist in clinical decision-making processes and improve patient outcomes.

SEVS AS BIOMARKERS FOR CANCER LIQUID BIOPSY

Several studies have investigated the potential of utilizing specific sEV protein biomarkers for diagnosing cancer using patients' samples. Promising sEV biomarkers have been identified for pancreatic cancer. A cell surface proteoglycan, glypican-1 (GPC1) is enriched in sEVs. GPC1 can be detected in serum of patients with pancreatic cancer, demonstrating an area under the curve (AUC) of 1.0 and a sensitivity and specificity of 100%.^[25] However, GPC1 is not exclusive to pancreatic cancer cells. It has been reported that colorectal cancer cells also overexpress GPC1.^[26] This indicates that GPC1 alone may not be sufficient as a standalone biomarker for pancreatic cancer, and the combination of several protein biomarkers may be necessary to achieve both sensitive and specific detection of pancreatic cancer. A detection panel consisting of sEV GPC1, sEV cluster of differentiation 82 (CD82) and serum carbohydrate antigen 19-9 (CA19-9) exhibit a promising diagnostic result of AUC of 0.942 to effectively differentiate healthy individuals from pancreatic cancer patients.^[27] It worth considering the inclusion of multiple

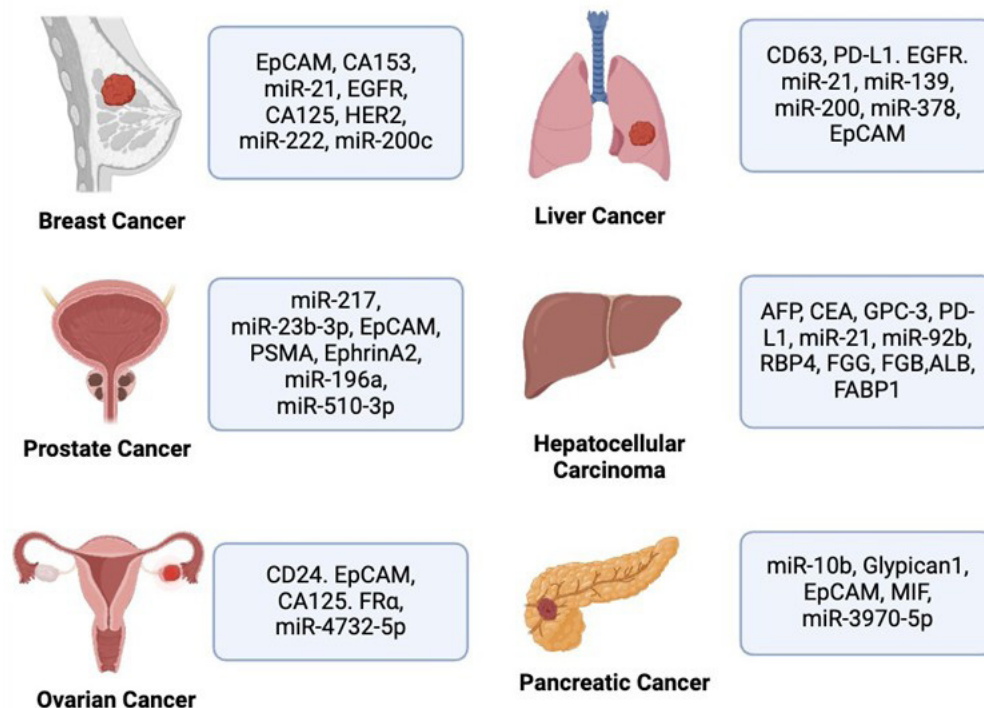


Figure 1: sEV biomarkers associated with several prevalent types of cancer. sEVs derived from cancer cells have emerged as a promising tool in the field of cancer liquid biopsy. They carry unique sets of proteins and nucleic acids that exhibit distinct expression patterns compared to non-cancerous cells. The identification and analysis of these exosomal biomarkers offer opportunities to develop precise diagnostic and prognostic tests. sEV: small extracellular vesicles.

biomarkers that are found to be overexpressed in pancreatic cancer cells in a diagnostic test. For example, miRNA-10b,^[28] mir-155,^[29] mir-125b-5p^[30] have been identified as overexpressed in pancreatic cancer and may serve as valuable additions for a more comprehensive assessment of the disease.

Epithelial cellular adhesion molecule (EpCAM) is of interest in cancer research due to its altered expression in various types of epithelial cells. The overexpression of EpCAM is also detected in other cancer types, such as breast cancer,^[31] ovarian cancer^[32] and prostate cancer.^[33] This highlights the necessity to include multiple biomarkers for increasing the accuracy of diagnostic tests. Biomarkers such as sEV FR α , sEV CD24, and sEV EpCAM have been employed to differentiate ovarian cancer patients from the control group. While FR α levels are nearly undetectable in control samples and substantially lower in sEVs from ovarian cancer, CD24 and EpCAM are both well-studied biomarkers. For sEV CD24, sEV EpCAM, and sEV FR α , the AUCs are 1.00, 1.00, and 0.995 respectively.^[34] The levels of three biomarkers can be taken into consideration together when diagnosing ovarian cancer. For accurate metastatic breast cancer diagnosis, eight sEV biomarkers are used, which includes CA 15-3, CA 125, carcinoembryonic antigen (CEA), human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), prostate-

specific membrane antigen (PSMA), EpCAM and vascular endothelial growth factor (VEGF). The combination of these biomarkers demonstrates a high accuracy with an AUPRC of 0.9912 in distinguishing breast cancer patients from healthy control utilising sEVs.^[35] There are still limited studies on diagnosing cancer from different cancer types due to the scarcity of exclusive biomarkers specifically expressed in a cancer type. Combining multiple biomarkers to diagnose cancer provides a more accurate result.

Tumour-derived sEVs actively communicate with the surrounding microenvironment through the expression of specific biomarkers. It is of utmost importance to identify these biomarkers, as they can serve a variety of purposes including predicting overall survival, assessing the risk of recurrence, classifying cancer stages and subtypes, and evaluating treatment resistance.

Predict outcomes and monitor progression

sEV biomarkers provide valuable insights into disease progression and survival rates in HCC. HCC patients with larger tumours or at later TNM stage have been found to exhibit lower blood sEV miRNA-638 levels, and those with these conditions additionally displayed poorer three- and five-year survival rates.^[36] This suggests the role of sEV miR-638 as a circulating cancer biomarker to predict poor prognosis for HCC patients. It has also

been discovered that sEV PD-L1 contributes to survival prediction. Shorter progression-free survival and overall survival are independently predicted by a rise in sEV PD-L1.^[37] The progression of cancer can be monitored by tracking changes in sEV biomarker profiles overtime. sEV biomarkers can also be used to determine the likelihood of cancer recurring after initial treatment. For example, significant differences in miR-718 expression were observed in serum sEVs of HCC cases with recurrence following liver transplantation compared to those without recurrence.^[38] This facilitates the identification of patients who require liver transplantation and aids in predicting HCC recurrence after surgery. sEV lncRNAs display disease-specific expression patterns, making them useful biomarkers for evaluating recurrences. To differentiate patients with recurrent colorectal cancer from those without recurrence, a 5-sEV lncRNAs panel was created.^[39] Another study also utilized serum sEV lncRNAs in breast cancer for recurrence prediction.^[40] The execution of an aggressive follow-up approach for patients who are at high risk of recurrence can be justified by stratifying patients based on their risk of recurrence.

Classify stages and subtypes

sEVs contains biomarkers which contribute to the staging of cancer by providing information about the tumor's size, invasiveness, and spread. This information aids in determining the appropriate treatment approach and prognosis for patients. Plasma sEV Sox2ot has been shown to correlated with TNM stage. Sox2ot is crucial for inducing EMT and stem cell-like characteristics, and sEVs with a high concentration of Sox2ot can facilitate tumour invasion and metastasis.^[41] Another biomarker, sEV TGF-B1, has shown a strong correlation with TNM. Patients with advanced gastric cancer, defined as TNM stages 2, 3, or 4, have demonstrated increased levels of sEV TGF-B1 compared to patients with stage 1 disease.^[42] This indicates that monitoring the expression level of sEV biomarkers can be a useful tool in classifying the TNM stage.

sEV biomarkers also play a crucial role in classifying cancer into different subtypes. In the context of breast cancer subtypes, patients with HER2- and HR+ tumours have better prognoses than those with more aggressive triple-negative (ER-PR-HER2-; TNBC) or HR-HER2+ malignancies.^[43] sEV miRNAs have been reported in these subtypes, with miR-335, miR-422a, and miR-628 showing significant differences between TNBC and HER2-positive individuals. The AUC values for miR-335, miR-422a, and miR-628 are of 0.737, 0.655, and 0.759 respectively. These sEV miRNAs have a sensitivity of 65% and 68% and a specificity of 84% and 81%, respectively, for differentiating between TNBC and HER2-positive individuals.^[44] The classification utilizing sEVs can assist in tailoring treatment

strategies to specific cancer subtypes.

Assess resistance in cancer

Chemotherapy, radiation, immunotherapy, and surgical excision are commonly used treatment modalities. Nonetheless, it is not uncommon for cancer patients to develop primary or acquired medication resistance, and emerging evidence suggests that sEVs may play a role in the dissemination of drug resistance. It was discovered that cisplatin-resistant cells and stomach cancer cells have elevated levels of sEV circ-0063526. This Cir-0063526 has been shown to be packaged in sEVs and delivered to sensitive cells, thereby promoting resistance to cisplatin. In patients with gastric cancer, high expression of sEV circ-0063526 has been associated with a poor response to cisplatin treatment.^[45] Similarly, differential expression of several microRNAs (miR-425-3p, miR-1273h, miR-4755-5p, miR-9-5p, miR-146a-5p, and miR-215-5p) has been observed, with the highest fold change in platinum-resistant non-small cell lung cancer (NSCLC) patients when compared to platinum-sensitive NSCLC patients. In patients with NSCLC, high miR-425-3p has been identified as a powerful prognostic biomarker for low responsiveness to platinum-based chemotherapy.^[46]

More than half of cancer patients use radiotherapy to treat localized cancer, relieve symptoms, or slow disease progression. Nonetheless, radioresistance continues to be the primary cause of radiation failure. A comparison between radioresistant and radiosensitive NSCLC patients revealed that sEV miR-96 was significantly overexpressed in the radioresistant group. An AUC value of 0.7496 was obtained for sEV miR-96's radioresistance diagnostic capability, suggesting its potential as a useful biomarker for distinguishing patients with radioresistant NSCLC from those with radiosensitive NSCLC.^[47]

Recently, immunotherapy has gained popularity as a treatment for cancer, encompassing various strategies such as immune system modulators, cancer vaccines, and immune checkpoint inhibitors. sEVs have been discovered to be a useful biomarker for choosing the most suitable patients for immunotherapy. sEV PD-L1 expression has been shown to be higher in patients receiving immune checkpoint inhibitor treatment. Its expression can be used to predict an overall survival of NSCLC patients. Patients who demonstrated a fold change in sEV PD-L1 expression equivalent to or higher than 1.86 exhibited a higher progression-free survival rate.^[48] This suggests that sEV PD-L1 expression can help identifying NSCLC patients who are likely to benefit from immune checkpoint inhibitor therapy and have a more favourable prognosis. Moreover, a prognostic and diagnostic model using two sEV-derived

Table 1: Clinical trials utilising sEV biomarkers.

Title	ID	Dates	Purpose	Conditions or diseases
Development of a prognostic and predictive biomarker for locally advanced breast cancer patients treated with neoadjuvant chemotherapy using sEVs	NCT05955521	Study start date: May 1, 2021 Estimated study completion date: July 1, 2028	Prognosis	Triple negative breast cancer and HER2-positive breast cancer
Clinical relevance of detecting molecular abnormalities in glial tumor sEVs	NCT06116903	Study start date: December 15, 2023 Estimated study completion date: December 15, 2025	Diagnosis	Glioma
Interrogation of sEV-mediated intercellular signaling in patients with pancreatic cancer	NCT02393703	Study start date: March 2015 Estimated study completion date: March 2025	Prognosis	Pancreatic cancer and benign pancreatic disease
Molecular profiling of sEVs in tumor-draining vein of early-staged lung cancer (ExOnSite-Pro)	NCT04939324	Study Start Date: June 21, 2021 Estimated study completion date: June 6, 2024	Diagnosis	Non-small-cell lung cancer
A companion diagnostic study to develop circulating sEVs as predictive biomarkers for the response to immunotherapy in renal cell carcinoma	NCT05705583	Study start date: January 1, 2023 Estimated study completion date: December 31, 2025	Prognosis	Renal cell carcinoma
A prospective, multicenter cohort Study of urinary sEV lncRNAs for preoperative diagnosis of lymphatic metastasis in patients with bladder cancer	NCT05270174	Study start date: June 1, 2023 Estimated study completion date: August 1, 2025	Diagnosis	Bladder cancer
Study of sEVs in monitoring patients with sarcoma (EXOSARC)	NCT03800121	Study start date: November 19, 2018 Estimated study completion date: November 19, 2025	Prognosis	Sarcoma
A retrospective study to compare biomarker expression of sEVs derived from peripheral blood and primary lung cancer drainage pulmonary blood in lung cancer patients	NCT05587114	Study Start Date: October 13, 2022 Estimated study completion date: December 31, 2025	Diagnosis	Lung cancer
Early detection of pancreatic cancer: prospective study	NCT06388967	Study start date: March 15, 2023 Estimated study completion date: November 21, 2025	Diagnosis	Pancreatic cancer
A sEV-based liquid biopsy for the differential diagnosis of primary liver cancer	NCT06342414	Study start date: March 15, 2024 Estimated study completion date: March 15, 2025	Diagnosis	Hepatocellular carcinoma
Early detection of stomach cancer with a liquid biopsy based on exosomal micro-RNA	NCT06342427	Study start date: March 15, 2023 Estimated study completion date: March 15, 2025	Diagnosis	Gastric cancer

Note: The table provides information on the clinical title, ID, cancer type, purpose, and dates of the clinical trials. These trials focus on utilising sEV biomarkers to develop diagnostic or prognostic test for cancer. sEV: small extracellular vesicles.

genes, MYL6B and THOC2, has been developed. High expression of these genes was associated with greater expression patterns of immune checkpoint genes, such as PD-1, B7H, CTLA4, and TIM3, in patients with HCC.^[49] Patients whose immune checkpoint expression is higher may likely to benefit from immunotherapy as it can boost the immune response resulting in greater therapeutic benefits.

TRANSLATION OF SEVS IN CLINICAL TRIALS

In recent years, there has been significant progress in the translation of sEVs for cancer diagnosis, leading to the initiation of clinical trials. These trials are designed to evaluate the potential utility of sEV biomarkers in detecting and monitoring various types of cancer. Clinical

trials investigating sEV biomarkers aim to develop sensitive and specific diagnostic tools that can aid in early cancer detection, monitor treatment response, and track disease progression. The unique properties of sEVs, such as their stability in various body fluids (*e.g.*, blood, urine), make them attractive candidates for liquid biopsies. Table 1 showcases several ongoing clinical trials that are actively studying sEVs as diagnostic and prognostic markers. These trials are specifically aimed at confirming the reliability of sEV nucleic acids and proteins as reliable markers. However, it is important to note that clinical reports and data analysis are pending publication. These clinical trials are expected to pave the way for the development of non-invasive, easily accessible, and cost-effective diagnostic tools for various types of cancer.

While genetic profiling in human tumour analysis is increasingly used for enhancing cancer diagnosis, these methodologies come with limitations. For example, RNA sequencing fails to identify variants located in noncoding DNA regions,^[50] the production of short reads by next-generation sequencing techniques can pose challenges when sequencing genomes with complex repetitive regions.^[51] Researchers envision a future where personalized cancer management is facilitated through the analysis of information carried by sEVs. This could result in earlier detection of cancer, enabling timely intervention, more effective treatment strategies tailored to individual patients, and ultimately improved patient outcomes.

NEW METHODS FOR SEV ISOLATION AND ENRICHMENT

While sEV biomarkers hold promise for clinical use, there are several limitations that need to be addressed before their widespread implementation. One of the primary challenges is the high throughput isolation of sEVs. Currently, there is a lack of standardized and efficient methods for isolating sEVs from various body fluids in large quantities. Existing isolation techniques often suffer from low yield and variability, which can affect the reliability and reproducibility of results.

Precipitation, size-based separation, ultracentrifugation, and immunoaffinity are some of the conventional techniques widely used to isolate sEVs. Ultracentrifugation is a commonly employed technique, which applies strong centrifugal forces to the sample, separating sEVs from other particles according to their size and density. The procedure involves pre-processing the sample to pellet larger particles, debris, and cells at lower speed, followed by higher speed ultracentrifugation to pellet sEVs.^[52] Size exclusion chromatography separates particles according to their hydrodynamic size as they pass through a porous

stationary phase. It offers mild conditions for separation, reducing potential harm to sEVs and preserving their integrity.^[53] Precipitation polyethylene glycol (PEG) is another often employed method. By adding a high PEG concentration to the sample, sEVs aggregate and precipitate, allowing for further processing and purification.^[54] However, these conventional isolation methods are not only tedious and costly, but also limited to low-throughput applications and require specialised instruments. In recent years, emerging methods and technologies have addressed these limitations, enabling the rapid and convenient enrichment of sEVs (Figure 2).

Size-based isolation

Deterministic lateral displacement (DLD) is a technique employed in microfluidics for the separation of particles within the nanometre to micrometre size range. It utilizes a series of bifurcations in the laminar flow pattern created by an array of regularly spaced pillars. This innovative method enables the efficient separation of particles from biological samples.^[55] Larger vesicles were laterally displaced across the array and collected at a side channel, while smaller vesicles flew out of the array in a zigzag pattern, thereby achieving the collection of sEVs. This resulted in the production of a nanoscale DL that can separate particles between 20 and 110 nm. This method demonstrates the size sorting of sEVs and enables fast colloid sorting in a continuous flow with single-particle resolution, paving the way for on-chip separation and diagnostics.^[56] The use of nanoscale DLD also has been proved to successfully isolate extracellular vesicles from serum and urine samples.^[57] Using double linked harmonic oscillations, EXODUS, an ultrafast isolation technology, combined two membrane filter configurations. Larger sEVs stayed in the central chamber while smaller particles and fluids were able to flow through the nonporous anodic aluminium oxide membrane due to periodic negative pressure oscillations caused by switching between periods of negative pressure and air pressure.^[58] The low yields and membrane pore blockage issues with the conventional approaches are resolved by these techniques.

Magnetic beads immunization

The utilization of magnetic bead-based immunoaffinity enrichment has gained significant interest due to its notable advantages in terms of convenience and high efficiency. This method involves the use of magnetic beads coated with specific antibodies that target surface markers of sEVs. Through this immunomagnetic approach, the process of capturing and enriching sEVs becomes more efficient and effective. Through the application of magnetic beads that specifically bound to the CD63 protein on sEVs derived from serum of mice having breast cancer, a nanodevice was able to separate target sEVs. The target sEVs could be eluted from magnetic

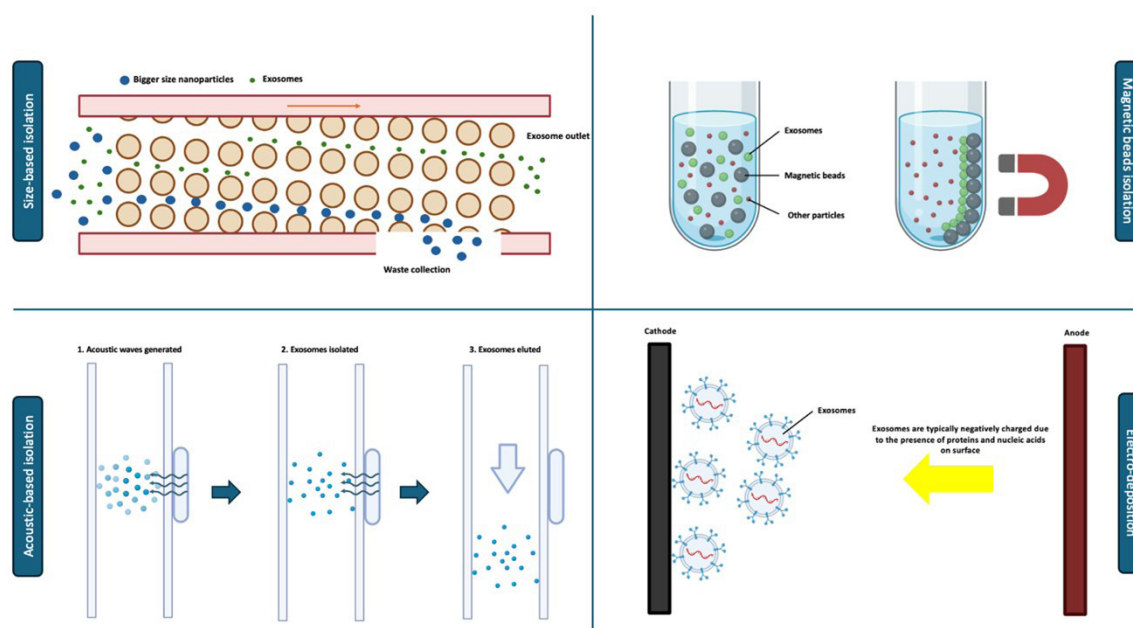


Figure 2: New methods for sEV isolation and enrichment. The figure shows four new exosome isolation and enrichment methods. **Size-based isolation** separates sEVs based on their size and large particle are excluded. **Magnetic based isolation** captures sEVs using magnetic beads conjugated with antibodies specific to exosome surface markers. **Acoustic based isolation** isolates sEVs using acoustic waves without the need of labelling. **Electro-deposition** separates sEVs via applying electric field across a sample and sEVs migrate to the oppositely charged electrode due to their negative charge. sEV: small extracellular vesicles.

beads by controlling light excitation while preserving their integrity.^[59] The problem of costly antibody applications on the beads is resolved by the aptamer approach. sEV-containing solutions can be selectively recognized and CD63 positive sEVs can be isolated using beads linked with CD63-1 aptamer.^[60] In another approach, magnetic beads conjugated with a synthetic peptide, Vn96, were used to isolate sEVs from MCF7 cell culture medium. This method achieved high efficiency in isolating sEVs without affecting their morphology.^[61] Numerous research endeavours have been dedicated to enhancing the functionality of magnetic beads. According to one study, immunomagnetic hedgehog particles (IMHPs) with nano-spikes can improve antibody-antigen based sEV binding and targeting by offering a greater surface area for immobilization of antibodies. These IMHPs demonstrated a capture efficiency of 91.7% in extracting sEVs from MCF-7 cells.^[62] These advancements in magnetic bead-based immunomagnetic techniques contribute to the effectiveness and functionality of sEV isolation.

Electro-deposition

Electro-deposition is a technique that leverages electrostatic forces to isolate sEVs based on their electrical properties. These forces facilitate the movement of charged sEVs towards a conductive electrode, which can be modified to enhance their adhesion. By selectively depositing sEVs onto the conductive electrode, this method enables effective

isolation and subsequent analysis and characterization of sEVs.^[63] For the purpose of isolating superparamagnetic nanobeads, a superparamagnetic track-etched membrane has been created. These beads possess a high capture capacity, short incubation time, and achieve capture rates of up to 99%, making them a viable option for isolating sEVs from physiological samples.^[64] Improved detection sensitivity was demonstrated by an electrode modified with chitosan composite, ionic liquid, and multi-walled carbon nanotubes. Breast cancer cell-derived sEVs containing HER-2 and EpCAM were found with high selectivity and sensitivity. Additionally, this approach presents the possibility of multiplex diagnosis detection of several sEV biomarkers.^[65] Electro-deposition enables the precise isolation of sEVs while maintaining their integrity, minimizing any potential damage or alterations that may occur during the deposition process.

Acoustic-based isolation

Acoustic forces can be utilized for the separation of sEVs from biological samples as well. By generating acoustic waves, spatial pressure nodes and antinodes are created, effectively causing sEVs to migrate towards specific regions for isolation. Due to their smaller size and lower density compared to cells and debris, sEVs can be selectively separated from larger and denser particles. This enables the efficient isolation of sEVs using acoustic forces.^[66] An example of this is the Acoustic Separation

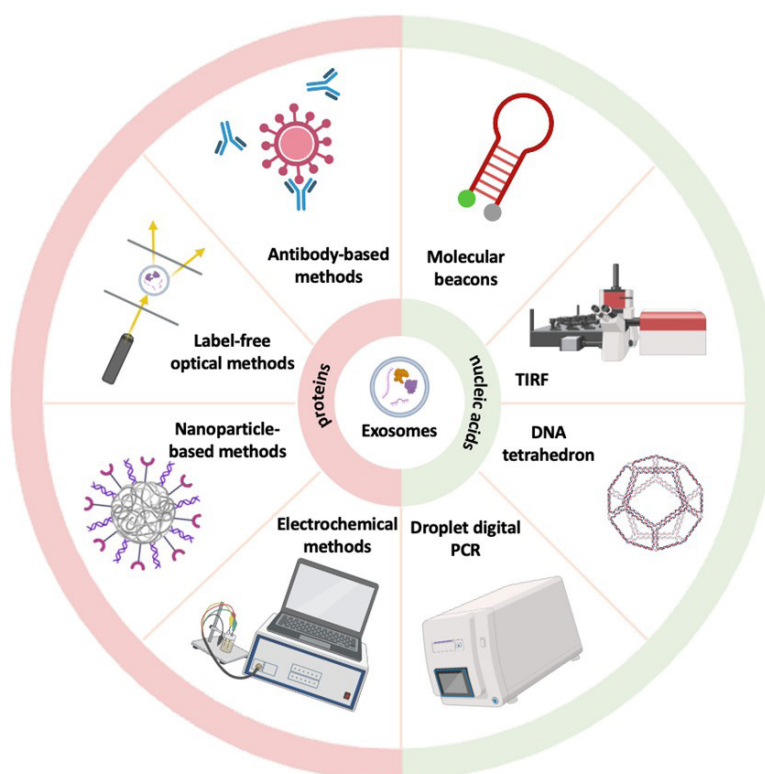


Figure 3: New methods for detection of sEV proteins and nucleic acids. The figure shows some new sEV proteins and nucleic acids detection methods, including antibody-based methods, label-free optical methods, nanoparticle-based methods, electrochemical methods for proteins, and droplet digital PCR, DNA tetrahedron, total internal reflection fluorescent and molecular beacons for nucleic acids. sEV: small extracellular vesicles.

and Concentration of sEVs for Nucleotide Detection (ASCENDx) device, which utilizes a rotating microfluidic disc to enrich sEVs. In order to enable centrifugation and fluid actuation within the microfluidic channels on the disc surface, surface acoustic waves and the fluid layer on which the disc floats can be coupled to form the acoustofluidic disc rotation. With excellent selectivity and specificity of 95.8% and 100%, respectively, the enriched sEVs demonstrated diagnostic potential for identifying circulating colorectal cancer miRNA biomarkers from patient plasma samples.^[67] From undiluted blood samples, sEVs can also be directly isolated using a different acoustofluidic technology. The platform consists of two sequential surface acoustic wave microfluidic modules: one for isolating sEVs and the other for removing cells. The sEV-isolation module purifies the sEVs by eliminating other EV subgroups, while the cell-removal module eliminates microscale blood components. This approach effectively yields high purity and quantity of sEVs from undiluted blood samples.^[68]

NEW TECHNIQUES FOR DETECTING SEV PROTEINS

Conventional techniques for detecting sEV proteins involve a variety of laboratory methods, including western blotting

and ELISA. However, these methods often have limitations such as limited sensitivity and difficulty in obtaining accurate and precise quantitative data.^[69,70] To overcome these limitations, some new techniques and methodologies have been developed in recent years (Figure 3).

Label-free optical methods

(SPR) is an optical sensing method that detects changes in the refractive index near a sensor surface caused by the binding of biomolecules, such as sEVs or their proteins.^[71] This technique has been used to precisely and sensitively detect HER2-positive sEVs. This technique offers a potential breast cancer diagnostic approach by differentiating patients with HER2-positive breast cancer from healthy individuals.^[72] This sensing approach can be expanded to accurately detect more sEV subtypes by simply altering the aptamer types. Utilizing sEV epidermal growth factor receptor and PD-L1 as biomarkers, SPR has also been used to diagnose lung cancer.^[73] Another analytical method for the identification and characterization of molecules is surface-enhanced Raman scattering (SERS). SERS uses chemical and electromagnetic processes to increase the Raman signal of tiny molecules affixed to the uneven metal surface.^[74] It has been used extensively in the search for sEV biomarkers. Using this method, a

portable Raman sEV assay for sEV detection and protein profiling was developed. SERS can be used to diagnose breast cancer, and biomarkers for HER2 and EpCAM have been found to have diagnostic potential on sEVs in plasma from patients with HER2-positive breast cancer. According to these proof-of-concept investigations, this assay could accelerate research on sEVs and open the door for the creation of innovative liquid biopsies for cancer monitoring and detection.^[75] It is possible to precisely identify sEVs generated from cancer cells by combining statistical pattern analysis with SERS. Through principal component analysis of the entire SERS spectra of the sEVs, lung cancer cell-derived sEVs were identified with 95.3% sensitivity and 97.3% specificity from sEVs originated from normal cells.^[76]

Antibody-based methods

The unique binding of antibodies to sEV surface indicators is the basis for antibody-based techniques such as fluorescence detection and lateral flow assays, which allows the detection and study of these markers in sEVs. The presence of target analyte is determined by antibody-antigen interactions in lateral flow tests. This technique has been used to detect isolated sEVs from a malignant melanoma cell line, with a detection limit of 8.54×10^5 sEVs/ μ L.^[77] Additionally, it has been used to create a point-of-care platform for the detection and tracking of colorectal cancer. Together with lateral flow experiments, CD147-containing sEVs were employed as a biomarker to identify and monitor colorectal cancer. This point-of-care tool was used to quantify the CD147 antigen embedded in sEVs that were isolated from plasma.^[78] By using fluorescent labelling, sEVs can be visualized using the fluorescent detection method. This method has been utilized to detect sEVs from biological samples. The estimated limit of detection for sEVs using this method was 1.29×10^3 particles/ μ L. Additionally, the difference in sEV concentration between sera of healthy individuals and cancer patients was evaluated.^[79] This technique could potentially be developed into a platform for the precise and specific identification of sEVs in biological samples for the diagnosis of cancer. A study showed that the fluorescence approach may reliably identify plasma sEVs, with an AUC of 0.85 for cancer diagnosis, to differentiate lung cancer patients from healthy persons.^[80] By employing other tumour-related sEV proteins as recognition targets, it is possible to isolate and identify sEVs from specific subpopulations derived from tumour cells, thereby improving the sensitivity and specificity of tumour diagnosis.

Nanoparticle-based methods

Colorimetric assays can be employed to detect sEVs using nanozymes, which are nanomaterials possessing inherent catalytic activity similar to those of enzymes. Through

surface modification with certain ligands, the nanozyme can selectively bind to sEV surface indicators. In a study, the CD63 aptamer was utilized to identify sEVs. The hybrid nanozyme's peroxidase activity was increased and a colorimetric signal was produced with the aid of the CD63 aptamer-bound sEVs. As a result, sEVs with a detection limit of 3.37×10^3 particles/ μ L could be found.^[81] Similarly, EpCAM aptamer was utilized in the nanozyme-based colorimetric assay to provide specific detection, hence enabling the differentiation of breast cancer patients from healthy individuals.^[82] Quantum dots (QDs), one of the several varieties of nanoparticle-based optical labels, are advantageous for sEV detection because of their small diameter (2–10 nm), which enables effective sEV labelling and detection in a smaller size range.^[83] Different surface protein markers on sEVs from various breast cancer cell lines were specifically and quantitatively detected using the QD-based technique, and cancer-associated surface protein indicators can be used to distinguish sEVs produced from cancer cells from normal sEVs. By employing QDs to analyse HER2 expression on plasma sEVs, HER2-positive breast cancer was identified. Patients with the disease had HER2 expression that was around five times higher than that of healthy donors, with an AUC value of 0.96875.^[84]

Electrochemical methods

Electrochemical Impedance Spectroscopy (EIS) is a method that assesses the impedance response of a system when subjected to an alternating current signal across various frequencies. This technique proves valuable in detecting impedance changes arising from the interactions between sEVs and electrode surfaces. Considering that sEVs possess distinct compositions, particularly in terms of their membrane and cytosolic charge-dependent contents, variations in their opacity can serve as distinguishing factors. Consequently, EIS enables the differentiation of sEVs derived from different cellular origins based on their unique characteristics.^[85] Electrochemical biosensors, on the other hand, utilize electrochemical reactions taking place at the electrode surface, which can be influenced by the presence of sEVs. In a recent study, tumour cell-derived sEVs were successfully detected using a combination of cyclic nicking enzyme cleavage and a hybridization chain reaction for dual-signal amplification. For this assay, a hairpin aptamer probe (HAP) containing an aptamer was designed. The aptamer specifically binds to PTK7, a protein found on the surface of sEVs, causing a conformational change in the HAP. This conformational change enables hybridization between the HAP and the linker DNA, initiating cyclic cleavage of the nicking endonuclease on the linker DNA. Consequently, sEV detection is transformed into DNA detection. By incorporating this approach with HCR signal amplification, the study achieved highly sensitive electrochemical detection of CCRF-CEM sEVs,

with a limit of detection as low as 1.1×10^4 particles/mL.^[86]

NEW TECHNIQUES FOR ANALYSING SEV NUCLEIC ACIDS

SEVs contain not only protein cargoes, but also nucleic acids, which have shown promising result as a specific biomarker for cancer diagnosis and prognosis prediction. To quantify the expression levels of sEV nucleic acids, techniques such as qRT-PCR, microarray, and next-generation sequencing have been widely used.^[87] However, qRT-PCR is limited to detecting nucleic acids with known sequences, while NGS is costly and involves complex library construction.^[88] Microarrays, although capable of analysing thousands of nucleic acids simultaneously, have low sensitivity.^[89] To address these limitations, efforts are underway to develop highly sensitive and convenient methods for sEV nucleic acid detection that overcome these challenges (Figure 3).

Molecular beacons

Molecular beacons are hairpin-shaped nucleic acid probes that detect specific target sequences. They form a stem-loop structure with a fluorophore and quencher in close proximity. When the target binds to the probes, the stem opens, resulting in the activation of fluorescence.^[90] It has been shown that miRNA-targeting molecular beacons can detect several miRNAs simultaneously in sEVs. In sEVs generated from MCF-7, molecular beacons hybridized with numerous miRNAs despite the high concentration of human serum.^[91] Even in the presence of human urine, molecular beacons were able to identify the markers miRNA-375 and miRNA-574-3p, which are present in sEVs produced by prostate cancer cells. This implies that they can be used to do liquid biopsies for prostate cancer using human urine.^[92]

Solid-state nanopore sensing

Solid-state nanopore sensing involves applying a voltage across a solid-state material that contains a nanopore. When nucleic acid passes through the nanopore, it temporarily blocks the flow of ions or electrons, causing a detectable change in the electrical current. This change in current can be measured and analyzed.^[93] This technique has been utilized to detect the only two cysteine-containing peptides from LRG-1, an emerging protein biomarker, that are uniquely present in the urine of ovarian cancer patients. The technique provided improved selectivity for detecting biomarkers in ovarian cancer.^[94] Moreover, solid-state nanopores have been explored as single-molecule counters for future digital diagnostic technologies, as evidenced by this technology's capacity to quantify more than six distinct microRNA concentrations.^[95]

Droplet digital PCR

In digital droplet PCR (ddPCR), the nucleic acid sample of interest is divided into numerous separate reaction droplets, each containing a few target molecules or none at all. These droplets undergo PCR amplification within a thermal cycler, and upon completion, fluorescence-based detection methods are employed to analyze the results. Each individual droplet is examined to determine whether it exhibits a fluorescence signal or not. By quantifying the number of positive and negative droplets, the absolute quantity of target molecules in the initial sample can be accurately calculated using Poisson statistics.^[96] A study used the ddPCR technology with sEV DNA to develop a sensitive and accurate approach for diagnosing tuberculosis. sEV and total DNA that was isolated from respiratory samples were targeted to the IS6110 region. The use of sEV DNA in ddPCR resulted in greater sensitivity and specificity of 98.0% and 76.9%, respectively, similar to whole DNA.^[97] This suggests that the combination of ddPCR platform with sEV DNA has the potential to provide a sensitive and accurate methodology for diagnosis of cancer. Another study evaluated the efficacy of various methods for detecting EGFR mutation in pleural fluid and plasma samples; the results showed that ddPCR in conjunction with sEV DNA had the highest sensitivity. With NSCLC patients, this method may be able to detect genetic alterations linked to resistance to EGFR inhibitor treatment.^[98]

DNA tetrahedron

DNA tetrahedron is a versatile DNA nanostructure that offers precise control over its architecture. Through chemical modifications and DNA self-assembly, it can be engineered to provide a wide range of amplified signal tags.^[99] A study used DNA tetrahedron nanoprobe (DTNP) based on fluorescence resonance energy transfer (FRET) to establish a sensitive detection approach for has-miR-146b-5p, a tumour-related microRNA. The target miRNA was intended to cause a structural alteration in the DTNP, leading to a significant enhancement in the FRET signal. This method facilitated the assessment of miRNA expression levels in various cell lines and demonstrated a low limit of detection.^[100]

Total internal reflection fluorescence

Total internal reflection fluorescence (TIRF) imaging assay relies on the principle of total internal reflection, where a laser beam is directed at a specific angle onto a glass or prism surface, creating an evanescent wave that penetrated only a short distance into the sample.^[101] With the use of this method, single sEVs and their miRNA contents in serum microsamples can be directly visualized and measured. Serum sEV miR-21 is a commonly used cancer biomarker.

The TIRF imaging test was used to analyse miR-21 and demonstrated a better performance than traditional real-time PCR assays in differentiating cancer patients from healthy individuals.^[102]

MICROFLUIDIC DEVICES UTILIZING SEVS FOR CANCER DIAGNOSIS

Microfluidic devices have been developed to integrate the isolation and analysis of sEVs into a platform where sEVs can be directly characterized and analyzed for downstream genomics and proteomics. Microfluidic devices can enrich sEVs from low-abundance sample for enhancing the sensitivity of downstream analysis. Tumour-derived sEVs can be directly quantified from as little as 1 μ L of plasma using an approach known as nanoplasmon-enhanced scattering (nPES), which also uses antibody-conjugated gold nanospheres and nanorods to capture sEVs. By detecting ephrin type-A receptor 2, this assay can differentiate between patients with pancreatitis, healthy individuals, and those with pancreatic cancer.^[103] Some microfluidic devices enable the identification and quantification of specific cancer-associated biomarkers carried by sEVs. The mRNA levels of MGMT (O6-methylguanine DNA methyltransferase) and APNG (alkylpurine-DNA-N-glycosylase), whose levels in tissue are inversely related to the effectiveness of drug treatment in glioblastoma multiforme, were examined in enriched tumour sEVs derived from blood using a microfluidic chip.^[104] By examining the expression and proteolytic activity of MMP14 on sEVs using three-dimensional nanopatterned devices, tumour growth and metastasis may also be tracked.^[105]

Microfluidic devices offer significant advantages for the rapid and portable cancer diagnostics using sEVs, making them suitable for point-of-care applications. These devices hold great potential for early cancer detection and monitoring treatment response. A nanoparticle-based biochip enables the capture of circulating sEV and enhances the fluorescence signals of encapsulated RNAs. This is achieved by a catalysed hairpin DNA circuit confined within cationic lipid-polymer hybrid nanoparticles tethered on the chip which amplifies these signals in situ, all in a single step. This biochip can selectively and sensitively identify low expression of glypican-1 mRNA in serum sEVs, allowing for the discrimination of patients with early- and late-stage pancreatic cancer from healthy individuals and patients with benign pancreatic disease.^[106] Another microfluidic device, using self-assembled three-dimensional herringbone nanopatterns, can identify low concentrations of tumour-associated sEVs in plasma. This device suggests sEV folate receptor alpha as a potential biomarker for early detection and progression monitoring of ovarian cancer.^[34] Microfluidic devices provide an efficient platform for utilizing sEVs as cancer biomarkers.

Their integration with isolation, enrichment, and detection techniques enables sensitive and specific analysis of sEV cargo, facilitating the development of non-invasive cancer diagnostic approaches.

CONCLUSION

sEV biomarkers have emerged as valuable indicators of cancer progression and treatment response. The utilization of sEVs in liquid biopsy enables non-invasive monitoring of disease and holds promise for personalized treatment strategies. Moreover, the translation of sEVs into clinical trials underscores their potential clinical utility. These trials aim to validate the diagnostic and prognostic value of sEV biomarkers, as well as explore their therapeutic potential in drug delivery and immunotherapy. Significant advancements have been made in sEV isolation and enrichment techniques, and in the detection of sEV proteins and nucleic acids. These advancements have improved the sensitivity and specificity of sEV-based diagnostics, allowing for more accurate and reliable detection and analysis of cancer-related biomarkers. Microfluidic chips utilizing sEVs have shown promise for cancer diagnosis, offering rapid and efficient analysis. These miniaturized devices integrate multiple functions into a single platform, enabling streamlined and high-throughput analysis of sEV biomarkers. However, further research and development are crucial to fully harness the potential of sEV biomarkers in clinical applications. Standardized protocols for sEV isolation, characterization, and analysis need to be established to ensure reproducibility and comparability across different studies and clinical settings. Additionally, long-term studies are required to evaluate the clinical outcomes and cost-effectiveness of integrating sEV biomarkers into routine clinical practice. In conclusion, sEV biomarkers have paved the way for a new era in cancer management.

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Informed Consent

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Conflict of Interest

The authors declare no conflicts of interest.

Use of Large Language Models, AI and Machine Learning Tools

None declared.

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Data sharing not applicable – no new data generated.

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