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To cite this article: Kamil Reza Khondakar *et al* 2023 *ECS Sens. Plus* **2** 043403

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Review—Prospects in Cancer Diagnosis: Exosome-Chip for Liquid Biopsy

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A liquid biopsy combined with an exosome-chip (EC) is an important detection tool for early cancer diagnosis. Exosomes have a crucial function in the exchange of information between cells and are present in biological fluids. ECs are miniaturized microfluidic devices designed to isolate, capture, and analyze exosomes for analysis of patient samples. Such devices offer on-chip detection, high-throughput analysis, and multiplex measurements. Further, these chips can integrate with electrochemical and optical detectors, and mass spectrometry enabling comprehensive studies of diseases. This review will cover the outlook on chip-based diagnostics for liquid biopsy, detection, and isolation of exosomes to support cancer diagnostics.

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Manuscript submitted July 5, 2023; revised manuscript received October 10, 2023. Published November 17, 2023.

Exosomes are extracellular nanovesicles and are actively released by the majority of living cells inside multivesicular endosomes through the creation of intraluminal vesicles.¹ They have nanometer-scaled size (50–100 nm in diameter), and exhibit a buoyant density ranging from 1.13 to 1.19 g ml⁻¹.² In 1997, exosomes were first discovered as an RNase in the budding yeast *Saccharomyces cerevisiae* and then were identified in a diverse array of mammalian cell classes.^{3,4} The core of the exosome comprises a ring structure of six proteins that belong to a similar class of RNases.⁵ Exosomes are released by all living cells into biological fluids including body plasma, urine, cerebrospinal fluid, and saliva, which are predominantly rich in membrane proteins.⁶ Exosome contains (ribonucleic acid) RNA species such as messenger RNA, microRNA, and other RNA's, lipid, and cell-specific proteins. Till now, 1639 mRNAs, 764 miRNAs, and 4563 proteins have been recognized in exosomes derived from various tissues.^{7–10} They significantly contribute to facilitating intercellular communication and the exchange of substrates, often inducing physiological alternates in target cells by transferring lipids, proteins, and nucleic acids. MicroRNA in exosomes can serve as a shuttle to transfer genetic materials between cells and regulate the gene expression to the target cells. Though many studies confirm that exosomes can interact with target cells,^{11–13} but the mechanism is still unclear.

Analyzing exosomes holds promise as a potential method for early cancer screening, providing valuable insights into the genetic makeup of tumors.¹⁴ This approach negates the need for invasive biopsies and holds promise for therapeutic applications.¹⁵ Furthermore, the study of exosomes plays a critical role in understanding the pathological aspects of cancer, neurodegenerative disorders, and various other diseases.^{16–19} Exosomes originating from malignant tumor cells are pivotal in shaping the tumor environment and facilitating the formation of pre-metastatic niches.²⁰ Notably, cancerous cells tend to release a higher quantity of exosomes bearing specific biomarkers on their surface compared to normal cells.²¹ Consequently, tumor-derived exosomes serve as a crucial biomarker for both the analysis and detection of cancer at different stages, from a pathological to clinical standpoint. Also, recent studies have established the utility of exosomes for cancer detection before and after treatment,^{12,22} where tumor-specific exosomes can provide a specific signature of transmembrane

protein,²³ and changes in exosomal protein may correlate after treatment.

Liquid biopsy has significant benefits compared to traditional tumor biopsies.²⁴ Body fluids including serum and plasma from cancer patients or urine, bronchoalveolar lavage fluid, synovial fluid, pleural effusions, amniotic fluid, saliva, etc. are widely employed for liquid biopsy.²⁵ Liquid biopsy was established to define circulating tumor cells, exosomes, and DNA present in the bloodstream of individuals with cancer.^{26,27} In addition, membranous extracellular vesicles, including nanoscale exosomes (<50 nm) and other vesicles actively secreted from cancerous cells, have been identified in cancer bloodstream. Further, liquid biopsy offers rapid screening, direct detection, tracking treatment response for cancer patients, and detection of minimal residual after surgery. Exosomes have emerged as a new type of biomarker that will offer potential merits for liquid biopsy of cancer cells using biofluids.²⁸ Being less costly and less risky than conventional tissue biopsies, a liquid biopsy can be performed much more frequently to provide up-to-date information about how a patient's cancer might be changing. Yoshioka et al., have developed a highly sensitive liquid biopsy method utilizing an ExoScreen tool to analyze circulating extracellular vesicles in patients with colorectal cancer.²⁹ It has been demonstrated that ExoScreen can be finished off within 2 h and requires a low amount (5 ml) of serum samples whereas the conventional method required 12 h to distinguish the presence of proteins in circulating extracellular vesicles and immoderate volume of serum samples are needed.^{29,30}

Protein biomarkers are invaluable tools for comprehending a wide range of diseases and finding applications in analytical epidemiology, screening, diagnosis, and prognosis.³¹ The quantification of soluble protein biomarkers, such as erbB-2 (epidermal growth factor receptor) in female breast cancer patients and prostate-specific antigen (PSA) in male prostate cancer patients, among others, has shown potential in the study of various cancer types.^{32–36} However, these methods often encounter issues related to specificity, accuracy, reliability, extended processing times, and high rates of false positives, primarily due to the nonspecific binding of protein biomarkers. In contrast, exosomes offer distinctive advantages for ongoing monitoring and enable selective detection because of their unique surface markers that can be targeted by antibodies and their characteristic transmembrane protein signature.³⁷ Various techniques have been employed for the detection of exosomes in biofluids. Nevertheless, due to the minuscule size of exosome nanoparticles, quantification poses a challenge. For instance,

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conventional methods like flow cytometry provide efficient sensing but may miss vesicles smaller than 200 nm due to weak light scattering, limiting the molecular insights gained.^{38–40} Other molecular assay methods, such as ELISA and western blot, often require substantial sample volumes and pose constraints regarding available specimens in biorepositories, making them impractical for clinical research needs. To address these limitations, recent advancements in sensor technologies based on electrochemical and optical detection mechanisms have been leveraged for the rapid, on-site quantification of exosomes in bodily fluids. Such technologies offer high sensitivity and selectivity with fast response times, addressing the challenges associated with traditional methods.^{41–44}

A lab-on-a-chip is a miniaturized platform that can perform many functionalities of a laboratory experiment onto a single chip which enables the precise manipulation and multiple analysis of analytes like various cancer biomarkers (cells, proteins, nucleic acids, exosomes, etc) for disease screening, monitoring, and diagnosis.^{45–47} In the context of exosomes, lab-on-a-chip technology can isolate, capture, and analyze these minute circulating vesicles directly from cancer patient samples. The microfluidic channels of the chip allow us for in situ analysis of the exosome samples from patients to understand the complexity of disease by investigating the clinical sample in a dynamic state. The concept of an exosome-chip (EC) is quite new to the lab-on-a-chip technology. Previous reviews^{48–50} have shown the emergence of EC as an innovative platform for exosome analysis providing versatile operations (screening, monitoring, detection) to be carried out on a single chip. Researchers have designed and fabricated various ECs to gain precious insights into diseases and potentially develop new diagnostic tools and treatments. This new generation ECs can transform healthcare diagnostics by providing precise exosome-based testing that provides vital information on cancer disease, and neurodegenerative disease, neuropsychiatric disorders.⁴⁸

In this review, we cover the current scenario of exosome detection from research to clinical diagnosis for cancer or other diseases. Exosomes are excellent biomarkers for early cancer diagnostics. In addition, this review covers an overview of isolation, separation, and various detection methods of exosomes for cancer cells encompassing prognostic evaluation as well as the early identification of disease occurrence. It will also cover the recent advancement in point-of-care devices for the detection of exosomes and their challenges. Figure 1 depicts some of the current and future advancements in EC technology. The future of EC technology has great potential as the integration of Artificial Intelligence (AI) and Machine Learning (ML) will revolutionize this field by providing rapid and precise exosome data analysis of individuals. These technologies can extract valuable information from exosomes about heterogeneity, shape, size, etc. as biomarkers by analyzing complex patterns of data that current technology fails to provide. AI-enabled ECs can significantly improve the treatment strategy for a new age of personalized medicine and provide a smart healthcare system.

State-Of-The-Art-Detection Of Exosomes

Modern exosome detection systems utilize cutting-edge microfluidic and nanotechnology systems for multiplexed investigation of these tiny vesicles. This cutting-edge chip-based technology provides insights into the compositions, shapes, sizes, functions, and prospective diagnostic uses of exosomes through high-resolution imaging and single-particle tracking. Here, we discuss some of the state-of-the-art systems for exosome analysis.

There are various methods developed to isolate, detect, and characterize exosomes for disease screening, monitoring, and diagnostics. The morphology and size distribution of exosomes can be used as distinguishing factors. Some of the well-known techniques are western blot assay, enzyme-linked immunosorbent assay (ELISA), nanoparticle tracking analysis (NTA), etc.^{49–58} Apart from that, transmission electron microscopy (TEM) and scanning

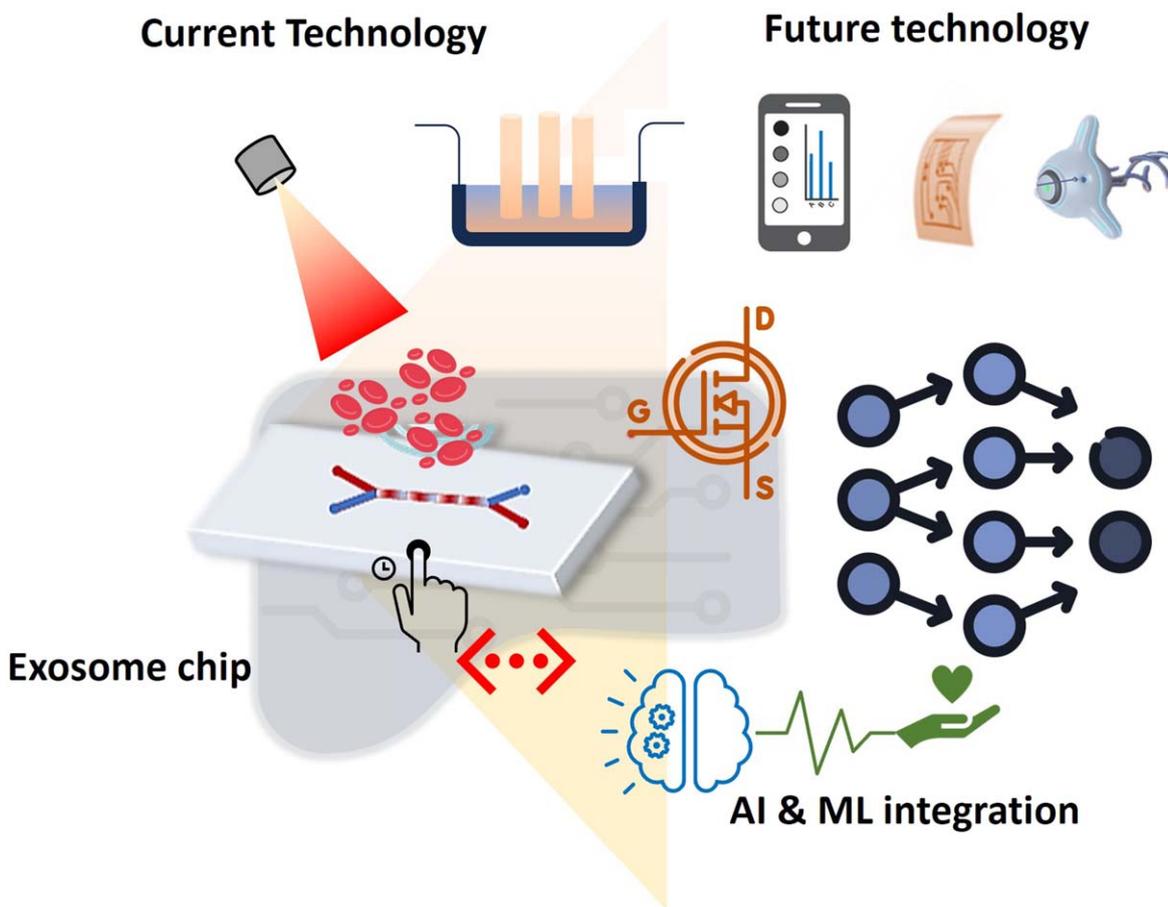
electron microscopy (SEM) have provided high-resolution images of exosomes to distinguish them from other extracellular vesicles.⁵⁹ Further, ECs have been explored by researchers to design versatile platforms for multiple analyses of exosomes. They could also provide morphology as well as size distribution of the exosomes for better clarity of extracellular vesicles for clinical application.

ELISA is one of the robust techniques to identify and confirm the protein receptors present in the exosomes.⁶⁰ These immunoassays use specific antibodies against exosome surface markers to capture and quantify exosomes for high-throughput screening and analysis. Another established technique is known as Western blot assay which is used to confirm the exosome samples containing the protein associated with the cell. Then the protein concentration extracted from the exosome is determined by a protein assay kit.⁶¹ Similarly, flow cytometry is another widespread technique for cell and exosome analysis. Multiple fluorescents labeled antibodies can be used to identify and quantify exosomes based on their surface proteins to determine the heterogeneity of the biomarkers for disease identification.

Exosomes can also be identified by NTA which is generally used for nanoparticle tracking.⁶² It uses laser light scattering to track and analyze the Brownian motion of the exosomes providing real-time information on the concentration and size distribution of exosomes. The other highly sensitive system is surface plasmon resonance (SPR) which can provide real-time and label-free screening of exosomes.^{63–65} This optical system works on the principle of total internal reflection which generates plasmons on the gold surface due to light-metal interaction causing a change in refractive index in the solution. This gold sensor chip will detect and quantify exosomes by monitoring changes in the refractive index upon capturing exosomes on a gold chip.

Microfluidic chip-based system has provided a versatile platform for exosome isolation, detection, and examination for clinical application. Some of the well-known chip-based methods developed for exosome analysis are viscoelastic flow sorting, acoustic nanofiltration, membrane-based filtration, immunoaffinity, trapping on nanowires, and deterministic lateral displacement (DLD) etc.⁶⁷ The purpose of developing these microfluidic systems is to achieve miniaturization, automation, and precise management of fluid flow, catering to exosome isolation and integrated analysis devices. Trau et al. introduced a SERS-based microfluidic chip for multiple exosome analyses from clinical samples to extract information for enhancing therapeutic outcomes and mitigating the advancement of drug resistance. (Fig. 2).⁶⁶ Furthermore, the researchers successfully extracted exosomes from samples of melanoma cancer patients. They then conducted a comprehensive analysis of multiple exosomes to gain insights into their heterogeneity and diverse phenotypic reactions to treatment. To minimize nonspecific adsorption, a nanomixing strategy was employed, which proves especially advantageous when capturing exosomes directly from complex biological samples.⁶⁶ Table I shows a list of different ECs with the sensing performances.

There are a few examples of exosome detection by using electrochemical sensor technology for early cancer diagnosis.^{68,69} A compact, eight-channel electrochemical sensor based on magneto-electrochemical assay was developed for efficient and real-time screening of exosomes at the site of collection.⁶⁸ In this sensor, the exosomes were anchored on magnetic beads surface (immunomagnetically) which were conjugated with horseradish peroxidase (HRP) enzyme, and beads were immobilized with antibodies (anti-CD63) specific to CD63 antigen (exosome marker). This electrochemical technology was able to detect extracellular vesicles in ovarian cancer patients' specimens. In addition, this sensor enabled the concurrent profiling of numerous biomarkers, overcoming the limitations of conventional assays in terms of limit-of-detection (LOD) and sensitivity. An electrochemical micro-aptasensor was developed for the early detection of exosomes from lung cancer patients by integrating a micropatterned electrode geometry and dual amplification.⁶⁹ This sensor provided a linear range of exosomes



AI enabled Exosome chip for a smarter healthcare ecosystem.

Figure 1. Schematic illustration of the current and future of EC technology.

spanning from 2.5×10^3 to 1×10^7 exosomes ml^{-1} and a LOD of 5×10^2 exosomes ml^{-1} . A cost-effective, electrochemical biosensor based on paper was developed to detect exosomes derived from ovarian cancer cells in cell culture media. This sensor consists of a sandwich-type immunoassay wherein the exosomes are captured by the electrode-bound anti-CD9 antibodies and subsequently identified through the presence of CA125, which is derived from ovarian cancer cells. This sensor provided a LOD of 7.1×10^8 exosomes ml^{-1} .⁷⁰ Unlike optical sensing, electrochemical sensing is a low-cost modality for the sensitive detection of exosomes owing to its considerable signal-to-noise ratio. Thus, electrochemical sensor technology could be an alternative point-of-care diagnostic tool for rapid examination of disease-specific exosomes in blood serum or other samples.⁷¹

Why Exosome-Chips are Important?

EC plays an important role in biomedical research due to its versatility. They are capable of rapid and efficient isolation, profiling, and analysis of exosomes from biofluids. Scientists are exploring their potential to uncover various functions of the human body as valuable biomarkers, and early disease detection mechanisms, and advance our understanding of intercellular communication and disease mechanisms.

There are several advantages of ECs. EC-enhanced liquid biopsies may be able to identify diseases at an early stage when conventional imaging or clinical tests may not be sensitive enough.^{79,80} Through prompt intervention and the beginning of treatment, this early discovery can greatly enhance patient outcomes. These chips enable longitudinal monitoring of illness development,

treatment response, and the formation of medication resistance or disease recurrence by the recurrent sampling of body fluids over time.^{72,81} This can offer insightful information for personalized medicine and treatment modifications. Further, ECs for liquid biopsies replace the need for risky and occasionally uncomfortable invasive tissue biopsies. Instead, the information can be obtained with a straightforward blood, urine, or saliva sample collection. Exosome technology is at a nascent stage with a lot of promise towards understanding disease biology, enabling early diagnostics, and developing targeted therapies for personalized medicine. However, ECs also have limitations. Some of the major limitations are the liquid biopsy sample complexity as they contain various extracellular vesicles, particles of similar size to exosomes that pose a huge challenge for isolation efficiency, low detection sensitivity (ultra-low concentration in the early stage of cancer), heterogeneous nature in size, surface markers, cargo, high cost of chips, less clinical testing of the patient sample, and so on.

Exosomes Isolation

There are several challenges for exosome isolation from body fluids due to its complexity in composition and functions. The composition of biofluids, such as blood or urine, can significantly impact the performance of detection techniques, especially affinity-based capture methods. The matrix effect refers to the impact of the complex mixture of proteins, lipids, nucleic acids, and other components present in biofluids on the accuracy and reliability of detection methods. The presence of these components can lead to various challenges, including, interference of molecules in biofluids that can lead to non-specific binding, interfering with the intended

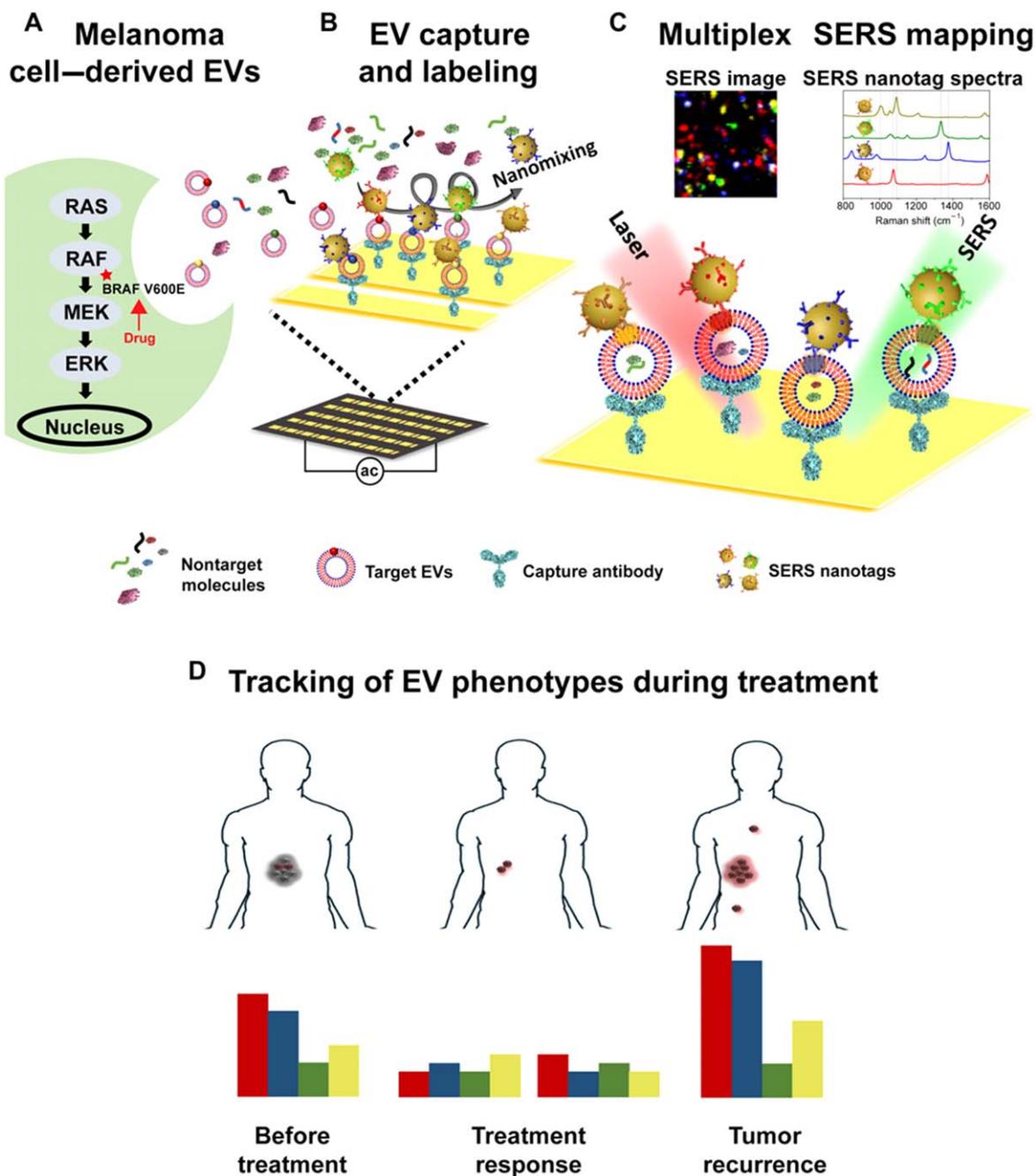


Figure 2. Schematic of a multiplex extracellular vesicles phenotype analyzer chip (EPAC).⁶⁶ (a)–(c) Capture of exosomes on EPAC from melanoma cells and multiplex EV phenotype analysis using SERS nanotags. (d) Examining EV samples before, during, and following BRAF inhibitor treatment enables the monitoring of phenotypic changes and provides valuable insights into treatment responses and early indications of drug resistance in melanoma patient samples. *Reproduced with permission.*⁶⁶

interaction between the target exosome biomarkers and the capture molecules, resulting in false positives or false negatives. Also, non-affinity-based biosensors which are based on physicochemical properties, structural changes, or other non-binding interactions face similar challenges. Therefore, when designing and optimizing detection systems, it is crucial to consider the effect of matrix in both affinity-based and non-affinity-based biosensors. Additionally, background noise can create a high baseline signal, making it difficult to distinguish the signal from the target exosome biomarkers, and can impact the sensitivity of the measurement.⁸² Consequently, addressing the matrix effect is crucial for improving the accuracy, specificity, and reliability of exosome detection methods. Sample preparation and pre-processing steps are essential stages for

exosome isolation for affinity and non-affinity-based biosensors. Generally, the common stages are centrifugation, filtration, or microfluidic platforms to remove or reduce interfering substances, such as proteins and lipids, from the biofluid samples (1–2). Furthermore, the integration of antibodies or aptamers can enhance the selectivity of the detection method, reducing the chances of false positives (3). Sometimes blocking agents can also reduce the interfering effects. Overall, a combination of careful sample preparation, tailored capture molecules, and innovative signal processing approaches is crucial in mitigating the matrix effect and enhancing the reliability of exosome detection methods in biofluids. Researchers working in the field of biofluid analysis are exploring innovative methodologies to mitigate the matrix effect and

Table I. List of different ECs with their sensing performances.

Name of chip	Techniques	Remark	References
Exosearch	Immunomagnetic beads	Multiplexed and quantitative isolation and release of exosomes from blood plasma.	Zhao et al. ⁷²
ExoPCD-chip 3D-nanopatterned chip	Electrochemical Three-dimensional self-assembled herringbone nanopatterns	On-chip isolation of exosomes from serum samples Ability of low concentration (10 exosomes per μl)	Xu et al. ⁷³ Zhang et al. ⁷⁴
ExoID-Chip	Fluorescence of photonic crystal using a filtration system	Detection limit of 8.9×10^3 EVs per ml and 20 μl sample required	Dong et al. ⁷⁵
ZnO-nanorods integrated (ZNI) microfluidic chip	Fluorescence	Limit of detection of exosomes (1.1×10^4 particles ml^{-1}).	Xu et al. ⁷⁶
Microarray chip device	Alternating current electrokinetic (ACE) & fluorescence	Isolate exosomes from undiluted human plasma specimen (30–50 μl) via on-chip analysis	Ibsen et al. ⁷⁷
Integrated microfluidic-SERS	Microfluidics integrated with surface-enhanced Raman spectroscopy (SERS)	Isolation and profiling exosomes from plasma (LOD = 2 exosomes ml^{-1})	Han et al. ⁷⁸
EV phenotype analyzer chip (EPAC)	Electrohydrodynamic force with SERS using nano-mixing fluid flow	Sensing cancer-specific EV phenotypes from melanoma patient plasma samples	Wang et al. ⁶⁶

enhance the precision of detection techniques.^{83–85} Therefore, extracting exosomes from clinical fluids can heighten the sensitivity of biomarker amplification and prevent unreliable outcomes. Conventional techniques including ultracentrifugation and density-gradient separation can segregate exosomes according to exosomes' buoyant density and size from clinical biofluids.⁸⁶ To effectively eliminate all larger entities, such as dead cells with a diameter exceeding $1\ \mu\text{m}$, the ultracentrifugation method employs a sequence of differential centrifugation steps at speeds of up to $200,000 \times g$. Nevertheless, this method has certain limitations. It does not yield highly pure isolates, and the process itself is time-consuming, typically taking 4–5 h. Additionally, it requires expensive equipment and has a relatively low recovery yield, ranging from 5% to 25%.⁸⁷ The density-gradient separation provides an improved recovery rate and purity over the ultracentrifugation. In the density-gradient separation method, samples are initially subjected to centrifugation in the presence of a viscous material with a density gradient. During this process, exosomes separate based on their isopycnic point, leading to their isolation. Nevertheless, this method is unable to effectively distinguish viruses' exosomes due to their similar buoyant densities. A schematic representation for exosome isolation using a ciliated micropillar array (Fig. 3). Recently, commercially available kits such as ExoQuick™ as well as Total Exosome Isolation™ were employed to isolate exosomes. Though these kits require only one or two steps and offer easy operation without using expensive equipment, they require an overnight incubation step.

Isolating exosomes from different cell sources, such as normal cells and cancer cells, can provide valuable insights into their distinct characteristics and potential diagnostic or therapeutic applications. Microfluidic systems offer a precise and efficient way to isolate exosomes based on their unique functionalities. Microfluidic devices are engineered systems that manipulate small volumes of fluids, such as blood or cell culture media, within microscale channels. They allow for precise control over fluid flow, enabling the separation and isolation of particles, including

exosomes, based on their size, surface markers, and other properties. Exosomes from normal cells and cancer cells can exhibit differences in their surface protein markers, cargo contents, and lipid compositions. These differences form the basis for their separation using microfluidic systems. Exosomes carry distinct surface proteins that reflect their cell of origin. For instance, exosomes from cancer cells might express tumor-specific antigens or markers associated with oncogenic processes. Microfluidic devices can be functionalized with antibodies targeting these specific markers. By flowing the exosome-containing sample through these devices, exosomes with the desired markers can be selectively captured onto the device's surfaces. Additionally, exosomes contain various molecules such as nucleic acids, proteins, and lipids. In the case of cancer cells, exosomes may carry specific biomolecules associated with tumor progression or metastasis. Microfluidic systems can exploit these unique cargoes for isolation. As an example, certain microfluidic designs incorporate affinity-based capture mechanisms, where ligands specific to cancer-related exosome cargoes are immobilized on the channel walls. When the sample flows through the device, cancer-derived exosomes bind to these ligands, allowing other components to be washed away. Besides, exosome membranes are enriched with specific phospholipids that can vary based on the cell source. Microfluidic systems can exploit these lipid differences by incorporating lipid-binding moieties on the device's surface. This enables selective adhesion of exosomes with specific lipid compositions, aiding in their isolation.^{88,89}

An affinity-based microfluidic device was developed to isolate exosomes.⁹⁰ In this device, the microfluidic chamber has a dimension of $19\ \text{mm} \times 4.5\ \text{cm} \times 20\ \mu\text{m}$ (H \times L \times W) along with $10\ \mu\text{m}$ deep herringbone grooves which increases the contact between the chip and the surface microparticles. After separation, the collected exosome particles are washed and characterized. In addition, an Exochip was developed to separate exosome particles. The device was constructed using a polydimethylsiloxane (PDMS) elastomeric material as well as incorporated functionalized antibodies specific to

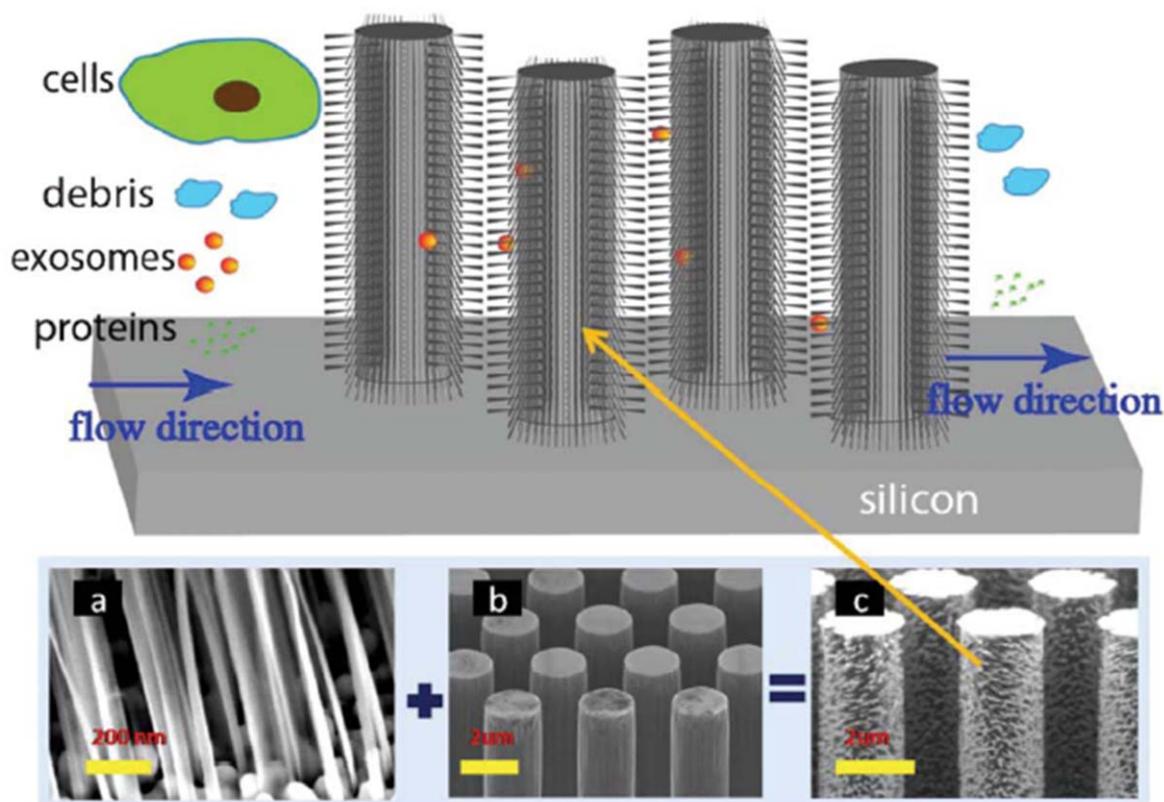


Figure 3. A schematic representation for exosome isolation using a ciliated micropillar array. This array is formed by a porous silicon nanowire-on-micropillar. A micropillar array used filter proteins, and cell debris from a liquid sample while exosomes were trapped within nanowires. *Reproduced with permission.*⁸⁸

CD63, an antigen commonly found in exosomes. Additionally, a fluorescent carbocyanine dye was employed as part of the device design.⁸¹ These ExoChips are excellent platforms for exosome-based diagnostic tools for human cancer molecular investigation and screening.

The isolation and analysis of exosomes have a range of clinical applications in contemporary cancer treatment. These include early-stage disease diagnosis, precision therapy, and treatment monitoring. This is primarily due to the high abundance of exosomes in bodily fluids, their accessibility through liquid biopsy, and the presence of nucleic acid and protein cargo derived from their specific cell of origin.⁹¹ There are quite a few techniques available to isolate or separate exosomes from complex biological fluids for liquid biopsy application. These separation techniques are based on certain factors such as sample volume, high purity, scalability requirements, and downstream analysis of the sample.

As defined above, ultracentrifugation stands as a widely employed method for the purification and isolation of exosomes. In this process, samples undergo high centrifugal forces, typically ranging from $100,000 \times g$ to $1,000,000 \times g$.⁹² This results in the formation of exosome pellets based on their sedimentation characteristics. The method involves a series of sequential centrifugation steps, each at an increasing speed, aimed at pelleting exosomes while eliminating cell debris and larger vesicles. Size-exclusion chromatography (SEC)⁹³ represents an alternative technique that capitalizes on the disparities in size between exosomes and other particles, allowing for their separation from complex biofluids. SEC employs a column packed with a gel containing porous material. Large molecules, unable to traverse the pores in the packing material, move through the column more swiftly and are the first to be eluted. In contrast, exosomes, being smaller in size, become trapped within the pores, which slows down their movement. Consequently, exosomes are separated based on their size using this approach.

Immunocapture techniques rely on the use of specific antibodies to capture, identify, and measure extracellular vesicles, including exosomes. This method has demonstrated remarkable efficacy in screening, diagnosing, and even predicting the progression of tumors in samples collected from cancer patients. In this approach, specific antibodies are affixed to solid surfaces, such as magnetic beads or plates. This immobilization allows exosomes to bind to the antibodies, serving as surface markers, and selectively capturing exosomes from patient samples. As highlighted by Logozzi et al., the benefits of this method extend beyond its adaptability to enable the concurrent assessment of multiple markers expressed on exosomes derived from diverse patient samples. These advantages also encompass cost-effectiveness and ease of implementation in nearly all clinical and biological laboratories worldwide.⁵²

Microfluidic-based isolation of exosomes is gaining attention for fast, reproducible, and automatable technology as it provides precise control over fluid flow and can be fabricated with specific patterns to selectively capture exosomes for low sample volume.^{90,94–96} Size-based filtration, affinity-based capture, and label-free isolation are some of the concepts being explored on chip technology for high-throughput and high-purity separation of exosomes. Chip-based technology is specifically beneficial for seamless integration with other detection systems for downstream analysis.

All the above-mentioned techniques have their pros and cons for exosome separation. However, each method has its advantages, limitations, and considerations for specific applications. Researchers are exploring and designing new separation techniques to tackle these challenges and trying to enhance the output and pureness of the isolated exosomes.

Some of the common technical challenges faced by scientists in exosome separation are expensive instruments with complex analysis, time-consuming, repetitive procedures, large sample volume, and low yield. One of the biggest challenges for the exosomes is addressing the heterogeneity within exosome populations.⁹⁷ These subtypes of exosomes are mainly due to the difference in size, functionality, biological origin, surface markers, cargo, and other

aspects.^{98,99} The presence of an impurity in exosome samples is very common as other biological analytes such as cell debris, proteins, lipoproteins, nucleic acids, etc are hard to separate. Even gold-standard techniques including ultracentrifugation cannot eliminate the impurities completely. Therefore, the removal of non-specific analytes is very crucial for exosome isolation.¹⁰⁰ Exosomes have similar characteristics such as microvesicles and apoptotic bodies, which poses challenges to achieving specificity and selectivity of the exosome sample. This is mainly due to the resemblance in size as well as the occurrence of analogous surface markers in all these vehicles which is difficult to extract pure exosome samples.¹⁰¹

Another issue in exosome isolation is its reproducibility⁹⁴ due to the dearth of standard protocols to maintain the quality control of clinical samples (blood, urine, saliva, and other body fluids). There is an abundant presence of proteins, nucleic acids, cell components, lipids, etc in plasma, serum, and urine samples of the patients which makes it a very challenging task to design a specific isolation protocol for exosomes. Apart from that, a few other challenges reported by the researchers are scalability issues for large exosome sample analysis along with preservation of the isolated sample for future analysis.

These exosomes provide myriad biological information including proteins, nucleic acids, and other biomolecules derived from their original cells.¹⁷ Without intrusive procedures like traditional tissue biopsies, it is feasible to learn more about the disease condition by extracting and analyzing exosomes from liquid samples using an EC. Exosomes from unhealthy cells, such as cancer cells or cells damaged by other diseases, can be found and examined on the chip for signs of disease. The exosomal payload, which may include proteins, nucleic acids, or other biomolecules, can be captured, and profiled by the chip to reveal details about the presence and features of the disease. Tumor heterogeneity is one of the biggest challenges in deciding disease diagnostics.¹⁰² Tumors frequently demonstrate tumor heterogeneity, with various tumor areas exhibiting different genetic mutations or protein expressions. ECs, which collect exosomes from biofluids, can offer a more thorough depiction of tumor heterogeneity than conventional biopsies, which are frequently restricted to a single tissue sample. The major advantages of ECs are as follows. (A) ECs can provide a rapid, simple, high-purity, and precise way for exosome analysis. (B) ECs can be used to track the effects of treatment over time. Real-time analysis of exosome cargo which act as carriers of biologically relevant molecules, including proteins and genetic material, can be indicative of treatment outcomes. Scientists can evaluate dynamic changes in the exosome cargo composition by analyzing EC at different time points during treatment.^{103,104} For example, the slight variations in specific proteins or genetic material associated with therapeutic targets or drug resistance mechanisms of any cancer patient exosome can provide the changes observed during treatment. (C) Further, exosome-based liquid biopsies can provide a less invasive option to conventional tissue biopsies as it will be a more practical and accommodating method for gathering diagnostic data.

The presence of such diverse biological information in exosomes presents an intriguing and cost-effective challenge for microfluidic technology. It also highlights the need for improved capabilities in terms of purity, diagnostic, and point-of-care acquisition. This review serves as a valuable resource for microfluidic experts, providing them with the opportunity to learn about this new class of biomarker-rich particles and the challenges associated with exosome enrichment. Additionally, biologists and clinicians familiar with exosome enrichment can evaluate the performance of novel microfluidic devices through this review.

Finally, it is noteworthy to mention that the exosome isolation from the complex environment of extracellular vesicles and bioparticles present in liquid biopsy samples is a difficult task. Due to the samples' heterogeneity, an isolation method that may selectively target exosomes while rejecting other vesicles and particles must be extremely specialized and effective. The similarity in size and surface characteristics of different extracellular components adds

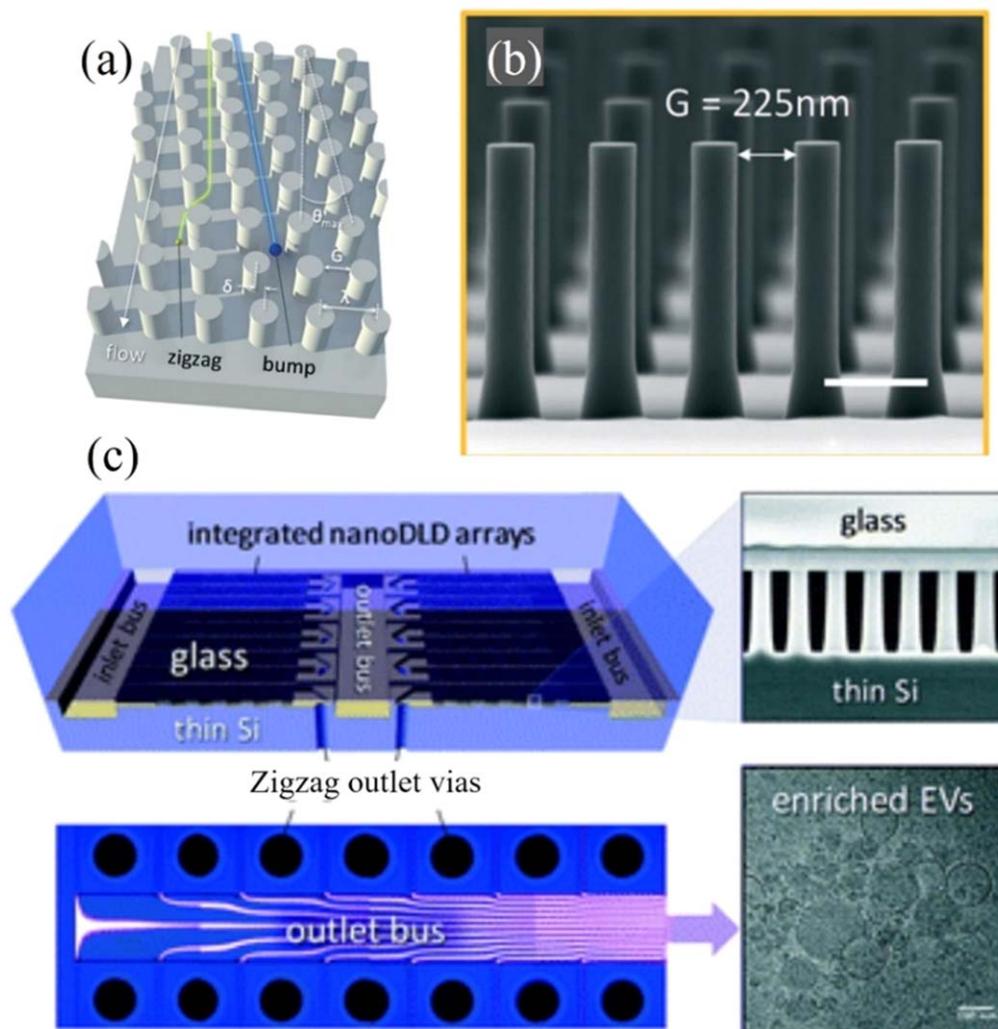


Figure 4. A size-based nano-DLD chip (22 mm × 30 mm) to separate exosome particles.⁹¹ (a) The schematic of the DLD chip features a pillar array with a specific gap size denoted as G . The pitch of the pillars, represented by λ , is approximately 400 nm. The row-to-row shift is indicated by δ , while the maximum geometric angle is denoted as θ_{\max} . The flow of particles within the chip is influenced by the array's geometry as well as the diameter of the particles being sorted. (b) A cross-sectional SEM image shows the pillar height, 1 μm , and gap, 225 nm (scale bar ~ 400 nm). (c) The integrated nanoDLD device with zigzag outlet and SEM images of separated particles. *Adapted with terms and conditions.*⁹¹

to this difficulty, necessitating creative approaches to identify exosomes in this complex environment. Exosome research and clinical diagnostics continue to face substantial challenges in the development of isolation techniques that guarantee high purity, yield, and repeatability while considering the inherently complicated nature of liquid biopsy samples. Therefore, a multidisciplinary strategy including expertise in cancer biology, biochemistry, microfluidics, and data analysis is necessary to address this challenge. More durable and dependable isolation techniques are anticipated to appear as technology develops and our understanding of exosomes grows.

Various High-Performance ECs for Exosome Analysis

Various high-performance ECs have emerged as powerful tools for exosome analysis. Such methods offer exceptional sensitivity, specificity, and scalability, making them invaluable for biomarker discovery, disease monitoring, and personalized medicine applications. Among the microfluidic-based ECs for exosome detection, the deterministic lateral displacement (DLD) platforms have been explored quite extensively for exosome analysis.^{105–107} These devices are highly efficient as they provide quick and automated

sample processing, cutting down analysis time and enabling high-throughput screening. Moreover, the easy integration of DLD devices with various characterization systems is gaining attention as a multiple detection platform for exosome analysis.¹⁰⁸ The DLD device consists of a fluidic platform that separates analytes based on their size and shape. The DLD chip is generally fabricated of micro or nanopillars or similar structures within a specific pattern inside a micro or nanochannel. The analytes which consist of exosomes, cell debris, nucleic acids, etc, have different shapes and sizes and are introduced in the channel using a combination of deterministic and inertial effects. Once the exosomes are inside the channel, they experience a hydrodynamic force that deflects them for a specific trajectory for separation from other analytes. Hydrodynamic force generates more force on the larger analytes like cell debris, and other vesicles deflecting them more from the main channel while exosomes being smaller in size are deflected less from the pillars. The exosomes get attracted to the pillars due to the size difference and fluid dynamics of the particles. The separated and extracted exosomes can be further utilized for downstream analysis involving a variety of characterizing techniques such as a nanoparticle tracking system for size distribution, a fluorescence-based technique for protein concentration, etc. In this way by integrating DLD platforms

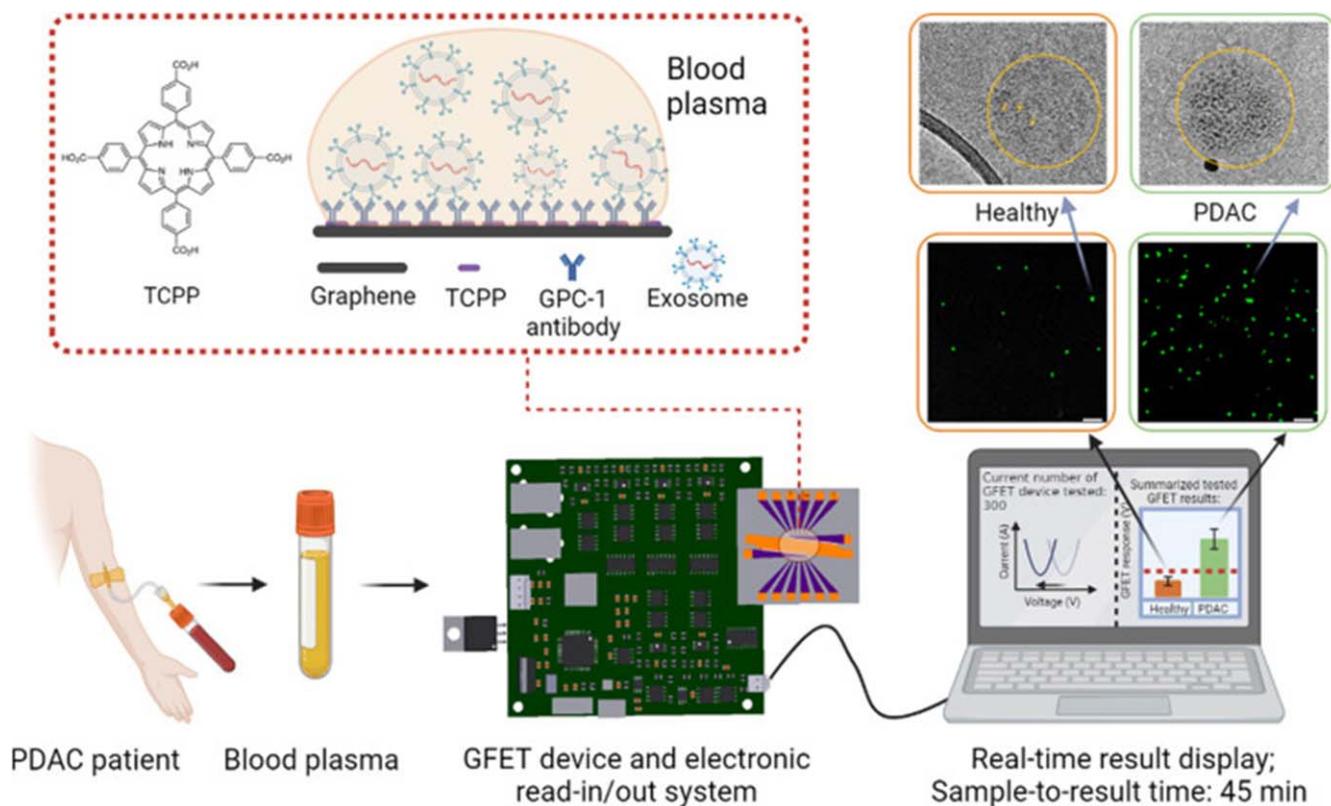


Figure 5. A graphene-based field effect transistor for rapid detection of pancreatic cancer exosomes. The detection time of this device is about 45 min. The graphene was functionalized with antibodies of TCPP43 and GPC-1 (zoomed-in images). Fluorescence images exhibited a greater number of exosomes on the graphene surface for the patient sample compared to a healthy sample. The TEM images showed more immunogold of the GPC-1 patient sample compared to healthy. *Adapted with terms and conditions.*¹⁰⁵

with other analysis systems, we can extract information from exosomes to understand disease conditions. In their study, Wunsh et al. developed nanoscale deterministic lateral displacement (nano-DLD) arrays with consistent gap sizes ranging from 25 to 235 nm. These arrays were designed to sort exosomes based on both size and surface markers. The goal was to enable on-chip sorting and quantification of exosomes directly from biological samples. (Fig. 4).¹⁰⁹ They demonstrated that this nano-DLD arrays-based chip can separate particle sizes from 20 to 110 nm with sharp resolution. In a similar vein, Smith et al. made further advancements by developing a system that incorporated nano-DLD arrays onto a single chip. This technology was specifically designed to separate exosomes from serum and urine samples.⁹¹ The isolated exosomes were analyzed for RNA sequencing obtained from patient serum samples for understanding the gene expression in prostate cancer.

Recently, graphene-based point-of-care devices are being explored for patient sample analysis. Yin et al. reported a graphene-based field effect sensor (GFET) platform for accurate and robust detection of pancreatic ductal adenocarcinoma (PDAC) in plasma samples obtained from patients through specific exosomes (GPC-1 expression). This new sensor consists of GFET sensor arrays with liquid gate electrodes integrated into the chip. A portable read-in/out electronic system was built to measure the real-time electrical response from the GFET sensors. This portable sensor can detect exosomes from plasma samples using a 20 μ l drop within 45 min. Authors claimed that GFET technology has the potential to differentiate between exosome samples obtained from healthy individuals and those with PDAC patients. They noticed that cancer-derived exosomes significantly bind to the graphene sensor surface as compared to healthy exosome samples of the same volume. For the clinical validity of the work, they compared their GFET results with standard non-invasive tools like magnetic resonance imaging (MRI) and computer tomography (CT) scan

data for early-stage pancreas cancer detection. Figure 5 describes the GFET sensor for point-of-care detection of pancreatic cancerous exosomes in patient plasma and demonstrates the test results in real-time display within 1 h. We need more such work in medical diagnostics for early-stage screening of cancer to reduce the mortality rate of the cancer. This portable sensor could replace the gold standard in cancer diagnostics as it demonstrated clinical testing in plasma samples.¹¹⁰

One of the interesting works for multiple exosome detection in clinical patient samples has been investigated by Zhang et al. The exosome profiling platform (ExoProfile chip) was fabricated with the 3D nanostructures of patterned colloidal self-assembly for exosome immunophenotyping. This ExoProfile chip is multiplex and allows multiple analyte detection. The validation of this chip was realized by purified exosomes from ovarian cancer cell line (SKOV3) and simultaneously detecting eight exosome biomarkers. The whole experiment and analysis were performed within 3 h using only 10 μ l ovarian cancer plasma. The circulating exosomes such as CA125, EGFR, CD24, HER2, FR α , EpCAM, and CD9 + CD63 were profiled from a number of plasma samples (~15 ovarian cancer patients) including 5 controls (benign). Importantly, they were able to distinguish seven exosome biomarkers from a panel of 20 samples and categorized both the early-stage and late-stage ovarian cancer patients based on their analysis and observed significant heterogeneity in exosomal expression among patients. Figures 6a–6c depicts the schematic illustration of the ExoProfile chip, working principle, and fabrication mechanism of the chip using microfluidic colloidal self-assembly.¹¹¹

Challenges and Prospects

Notably, ECs can produce different types of data such as size, concentration, detection, separation, and analysis of biomarkers of

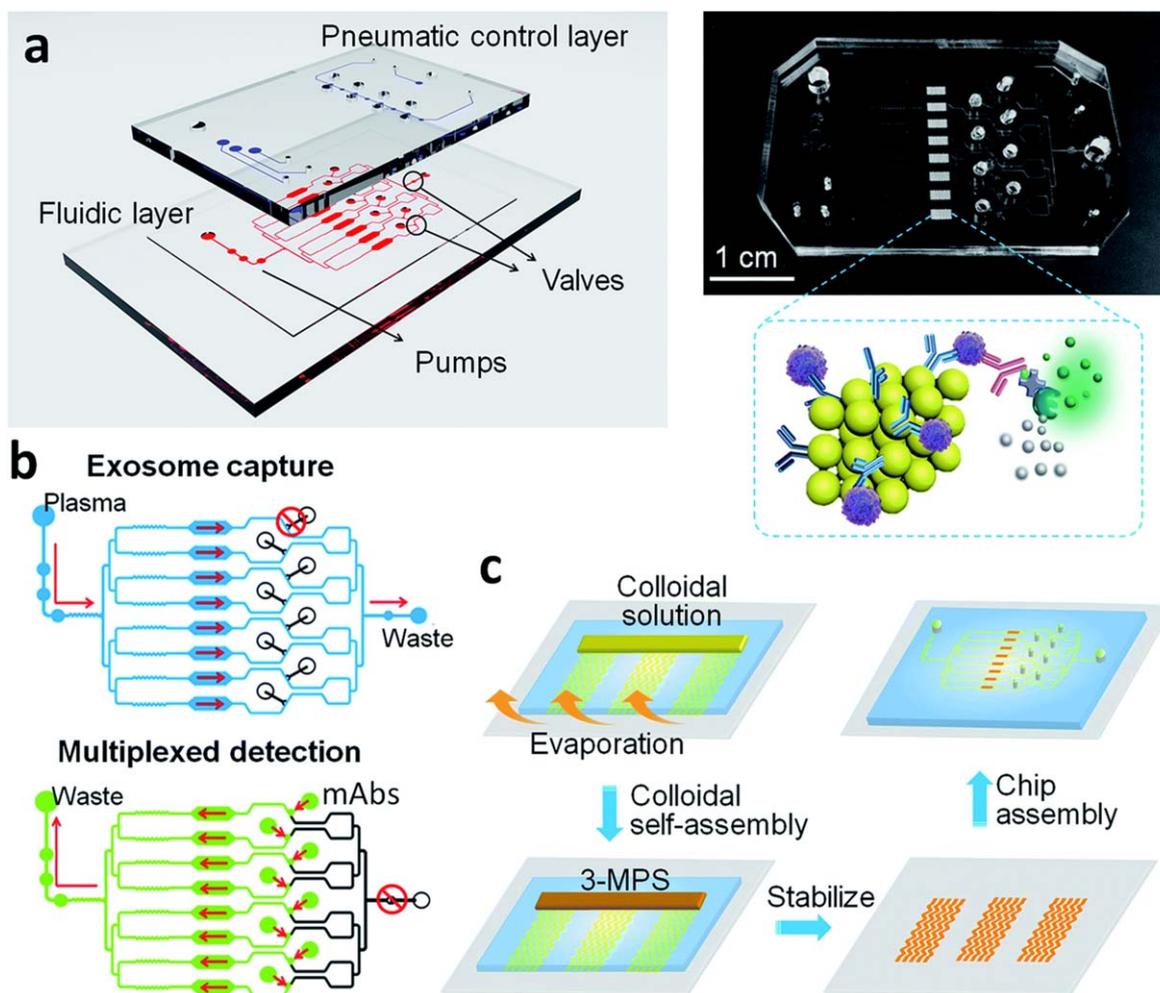


Figure 6. Schematics of ExoProfile chips. (a) An ExoProfile chip has a pneumatic and a fluidic layer along with 3D serpentine nanostructures and was used for the immunophenotyping of exosomes. (b) The ExoProfile chip along with flow manipulation showed an immuno-reaction and the multiplexed detection. The arrows showed the flow direction of exosomes. (c) Schematic presentation of the ExoProfile chip manufacturing. *Adapted with terms and conditions.*¹⁰⁶

many target diseases. There are two main skills such as data analysis and pattern recognition that can be used to analyze the produced data from an EC. Further, the data produced by ECs may be quickly analyzed by applying an ML algorithm to find a specific trend or pattern that might be connected to a target disease. This can help in the identification of new biomarkers and more precise illness detection. Noted that the ML algorithms can establish correlations in huge datasets that human analysts might overlook. In addition, AI may identify potential biomarkers that denote particular diseases by tracking patterns and connections, making liquid biopsy more focused and efficient. Using the patterns from various clinical datasets, AI can classify and separate exosome profiles into disease-specific classifications.

The ML algorithms can monitor changes in exosome profiles over time, assisting in the monitoring of therapeutic effects. This dynamic analysis or results from ML models can assist clinicians in speeding up patient treatments. Also, AI and ML algorithms can assist in lowering the number of false positives and negatives in generating liquid biopsy data by integrating additional patient data from previous records. Hence, the sensitivity and selectivity of the test can be enhanced drastically. This all-encompassing method may result in more precise diagnoses and individualized treatment plans. Another major area of future work could be predictive modeling for treatment strategies for better patient outcomes. AI can be utilized to create predictive models that forecast the course or recurrence of disease with the help of previous exosome data.

ECs are widely used as next-generation biomarkers in POC diagnostics due to ongoing research and emerging technology developments that continue to overcome various issues related to screening, monitoring, and therapeutic aspects. ECs can greatly improve the accuracy and sensitivity of exosome analysis at the point of care for early disease detection, exploring personalized medicine, and thereby, improving patient outcomes.

EC development and commercialization for POC applications face quite a few challenges. The fabrication of chips should be standardized, exosome capture efficiency and specificity should be improved, portable detection technologies should be integrated, and large clinical cohorts should be used for validation. To ensure reproducibility and dependability, standardization of chip manufacture, capture procedures, and detection techniques are required. Large-scale clinical investigations will be required to confirm the clinical applicability of ECs and evaluate the outcome in comparison to traditional diagnostic techniques.

So far, nanomaterials' structural innovation, including screening printing electrodes,¹¹² has proven enormous success in biosensor development.¹¹³ However, their performance and sophistication depend on the manufacturing modalities as the geometry of the electrode plays an important role.¹¹⁴ Traditionally, photolithography (i.e. micro/nanofabrication) and electrochemical deposition are two excellent manufacturing modalities widely used to develop electrochemical and SPR biosensors. The commercially successful glucose sensor is an extensively adopted screening printing-based

manufacturing modality to reduce cost per test.^{115,116} Further, the incorporation of nanomaterials for developing electrochemical ECs showed enhanced performance due to the high loading of antibodies (*anti*-CD63) specific to exosomes.¹¹⁷ The traditional EC developed for electrochemical nanosensing of exosomes (or antigens) provides limited sensitivities and limit-of-detection owing to the one and two-dimensional geometries of the electrodes with limited reactive surfaces.¹¹⁸ It has been realized that nanostructuring of sensor surfaces alone is inadequate to detect low target concentration down to the femtomolar level. Recently, additively manufactured three-dimensional (3D) microelectrode geometries of the electrochemical sensors overcome these limitations for detecting antigens or antibodies due to the larger surface areas of the electrodes. Unlike traditional manufacturing, 3D printing provides customizability, complex geometries, and multiplexity of sensor construction.^{119,120} A 3D micropillar array electrode called a “*multi-length-scale electrode*” of gold nanoparticles was coated with a thin layer of graphene to detect the attomole concentration of neurotransmitters.¹²¹ In this multi-length-scale electrode, the micropillar geometry of the electrode reduces the diffusion path of the target to interact with the electrode, while graphene accelerates the electrochemical reactions at the nanoscale due to their comparable size, thus accomplishing an attomolar sensitivity of a neurotransmitter (i.e., dopamine) detection.¹²¹ A similar mechanism was applied to build a 3D immunosensor to detect COVID-19 in seconds at a low concentration of antibodies (1 femtomolar).¹²² These applications demonstrated that the hierarchical architecture of 3D multi-length-scale electrodes opens the possibility of detecting target molecules including exosomes at ultralow concentrations.

There is increasing suggestion that ECs indicate cancer progression which will enable rapid and sensitive tools for quick analysis. As a result, current LOC techniques must be investigated to find better methods for isolating and locating malignant cell subpopulations that express proteins in a downregulated manner. In addition, by employing expression profiling to find organ-specific metastatic signals in exosomes, it might be possible to determine the tissue of origin of exosomes to determine the disease condition.

However, there are several challenges that must be overcome to commercialize EC technology. Though manufacturing of such devices may not be expensive, their standardized protocols for exosome isolation and analysis are still evolving, making it challenging to ensure consistent and reproducible results. It is noteworthy to mention that obtaining regulatory approvals for clinical use is a tedious and expensive process which is one of the major commercialization challenges of EC technology. Established liquid biopsy methods such as ctDNA analysis and circulating tumor cell (CTC) capture are already in the market, making it challenging for EC technology to gain market share.¹²³ Compared to these methods, EC technology offers higher sensitivity and higher speed of analysis. Exosomes are often more abundant than ctDNA or CTCs and, hence, improved sensitivity. Besides, exosomes carry a diverse range of biomolecules, providing a more comprehensive picture of the tumor’s biology. In addition, exosomes can be detected before ctDNA mutations become apparent.

The use of EC technology in cancer diagnosis raises ethical concerns. For instance, patients must be informed about the potential for incidental findings, as exosome analysis may uncover unrelated health issues. Highly sensitive tests may lead to the overdiagnosis of indolent cancers, raising questions about the need for treatment. Additionally, records of patient data, especially genetic information, require privacy safeguards.

Conclusions

This review article has covered the outlook of ECs with recent advancements for liquid biopsy. We have summarized various applications of ECs as diagnostic tools, exosome isolation, separation, and electrochemical detection. Unlike ultracentrifugation, though magnetic bead-based immuno-separation is an inexpensive

process, it is not feasible for cross-reactivity. With structural innovation in chip fabrication technology, various lab-on-a-chip platforms including POC sensors show huge potential for exosome analysis, however, there are still challenges to overcome. Integration of AI and ML with ECs would further improve cancer diagnostics for better patient outcomes. It is essential to optimize device design and operation parameters and validate through clinical investigations for high-precision ECs.

Acknowledgments

The authors gratefully acknowledge the partial financial support from the VT CALS Strategic Plan Advancement Seed Grant, CeZAP Pilot Grant and the 4-VA Pre-Tenure Faculty Research Award. The authors also thank Dr Hirak Mazumdar from Woxsen University, India, for assisting with Fig. 1.

Competing interests:

The authors declare that they have no competing interests.

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