

Review

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Review

Recent Advances in Microfluidic-Based Extracellular Vesicle Analysis

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Abstract: Extracellular vesicles (EVs) hold enormous potential in the diagnosis and treatment of diseases, benefit from their roles in intercellular signaling, as well as their plentiful presence and stability in body fluids. However, conventional EV isolation methods are labor-intensive, and harvest EVs with low purity and compromised recovery. In addition, the drawbacks, such as limited sensitivity and specificity of traditional EV analysis methods hinder the application of EVs in clinical use. Therefore, it is urgent to develop effective and standardized methods for isolating and detecting EVs. Microfluidics technology is a powerful and rapidly developing technology that has been introduced as a potential solution for the above bottlenecks. It holds the advantages of high integration, short analysis time, low consumption of samples and reagents. In this review, we summarize the traditional techniques alongside microfluidic-based methodologies for the isolation and detection of EVs. We emphasize the separation advantages of microfluidic technology in enhancing EV capture efficiency and precise targeting, while summarizing its analytical role in targeted detection and discussing the significance of EV detection in the tumor microenvironment, providing a direction for achieving automated and high-throughput EV detection in clinical samples.

Keywords: extracellular vesicles; microfluidics; EV isolation; EV detection

1. Introduction

Extracellular vesicle (EVs) are membranous particles enclosed by lipid bilayers with diameter ranging from 50 nm to 1 μ m derived from almost all cells [1]. By inheriting abundant signaling molecules from donor cells, such as nucleic acids and proteins, EVs play a role in intercellular communication and associate with disease progression [2–4]. EVs can be classified into several subgroups based on size and generation mechanism, including microvesicles, microparticles and exosomes [3,5]. Among these subgroups, exosomes are microvesicles ranging in size from 50 to 150 nm with an average of \sim 100 nm [3]. Multivesicular bodies (MVBs) contain multiple intraluminal vesicles (ILVs), which fuse with the plasma membrane to release ILVs as exosomes [6]. Therefore, exosomes contain proteins of cell surface and soluble proteins linked to the extracellular environment [7,8]. Efficient isolation and reliable analysis of EVs are important prerequisites for the clinical application of EVs. Conventional techniques for isolating EVs, such as ultracentrifugation, ultrafiltration, particle size exclusion chromatography, and polymer precipitation, are time-consuming and inefficient [9]. Microfluidic technology achieves efficient EV isolation and detection with only a very small amount (10^{-9} to 10^{-18} liters) of liquid specimens and reagents, and has the advantages of integration, high throughput [10]. With the support of microfluidic technology, the successful establishment of clinical EV detection platforms is expected soon. In this review, we will explore the latest developments in microfluidic-based EV isolation and analysis, as well as discuss the challenges and future directions of this rapidly evolving field.

2. Microfluidic-based EV isolation strategies

2.1. Label-free microfluidic isolation

Label-free isolation strategies depend on the physical characteristics of EVs, such as their size, density, and deformability, to separate them from other components present in bodily fluids. Conventional label-free methods, such as ultrafiltration (UF), ultracentrifugation (UC) and size-exclusion chromatography (SEC) usually require large sample volumes. UC differentiates EVs and other components based on size and density, and has been recognized as one of the most commonly used EV isolation methods [11,12]. However, the long UC period might result in coprecipitation of protein aggregates. In addition, the quality of EVs cannot be guaranteed due to repeated centrifugation processes and excessive centrifugal force [13]. UF adopts membrane filters with suitable pore sizes to purify EVs from other particles [14]. Regrettably, the accumulation of debris on the filter membranes will decrease the efficiency of EV isolation and shorten the lifespan of the membranes. [15]. SEC isolates particles with different sizes based on their different flow rates on the column filled with porous beads. As a mild EV isolation method, SEC can obtain EVs with relatively complete structure and function [16,17]. However, the tedious procedure, low-throughput and recovery of SEC limit its wide application.

Holding the properties of easy-integration, high-throughput and low sample consumption, microfluidics technology provides a variety of strategies for efficient label-free EV isolation [18–20]. For example, Li et al. integrated filtration and microfluidics technology to design cascaded microfluidic circuits for preprogrammed, clog-free and gentle isolation of EVs directly from blood within 30 min (Figure 1a) [21]. The problems of filter fouling and particle aggregations were solved by the pulsatile flows generated by the porous membrane, which lifting particles away from the membrane. Researchers engineered an automated centrifugal microfluidic disc featuring membranes with specific functionalities (Exo-CMDS) for exosome isolation (Figure 1b) [22]. As a one-step method, Exo-CMDS enriched exosomes with optimal exosomal concentration of 5.1×10^9 particles/mL from small amount of blood samples (<300 μ L) in 8 min. The above methods require tedious fabrication of microfluidic chips, which increased the uncertainty of trials. Liu et al. proposed a viscoelastic-based microfluidic system for direct label-free isolation of exosomes (Figure 1c) [23]. A simple microfluidic device with two inlets, three outlets and a straight microchannel was designed for viscoelastic separation of exosomes. The presence of polyethylene glycol (PEG) causes elastic lift forces which drives EVs toward the microchannel centerline according to their sizes. Under the size cutoff of 200 nm, exosomes were separated from large EVs. In addition, Yang et al. demonstrated a self-adaptive virtual microchannel for nanoparticle enrichment and separation in a continuous manner (Figure 1d) [24]. A gigahertz bulk acoustic resonator and microfluidics triggered and stabilized acoustic waves and streams to form a virtual channel with a self-adjusted diameter from dozens to a few micrometers. By a customized arc-shaped resonator, exosomes from patient plasma were purified. The system is stable and has high automation potential because of the self-adaptive and contactless continuous separation mode. Although label-free microfluidic isolation has the advantages of high throughput, rapidity and cheapness. The low purity and inability to isolate EV subtypes limit its downstream application.

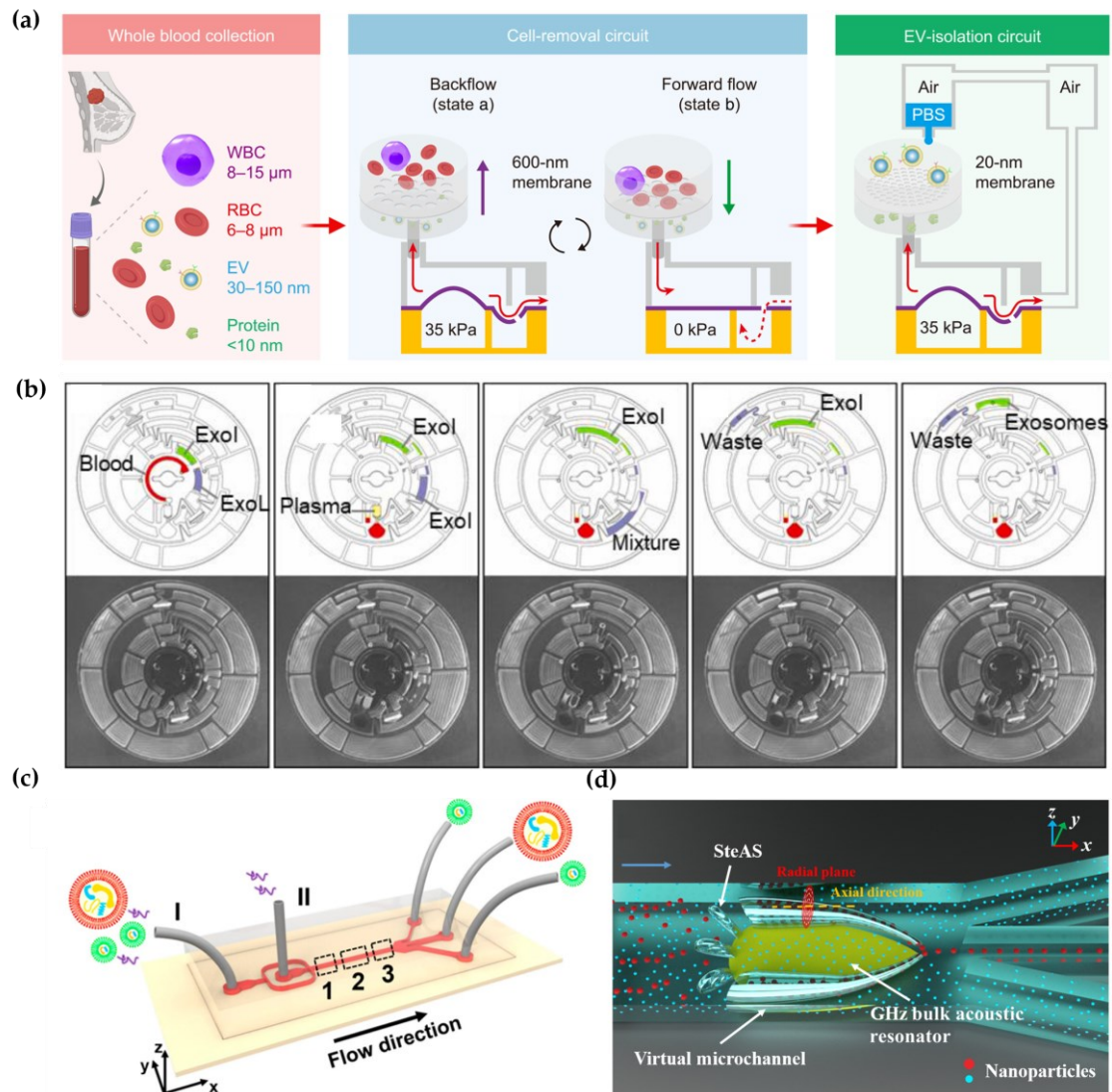


Figure 1. Label-free microfluidic-based EVs isolation methods (a) Cascaded microfluidic circuits for EV isolation. Reproduced from Ref. [21]. (b) Centrifugal microfluidic disc for EV enrichment. Reproduced from Ref. [22]. (c) Visual representation of the separation mechanism of viscoelastic microfluidics. Reproduced from Ref. [23]. (d) Mechanism of the stereo acoustic stream (SteAS) platform. Reproduced from Ref. [24].

2.2. Affinity-based EV isolation

Affinity-based isolation methods exploit the interaction between affinity ligands (antibodies, peptides or aptamers) and receptors on EV membranes to isolate EVs specifically [25,26]. These ligands are typically modified on the surface of materials or interfaces, such as magnetic beads [27–29], carbon cloth [30], graphene [31], or Ti2CTx MXene membranes [32]. The strength and duration of the interaction between affinity ligands and receptors on surfaces determine the capture efficiency of EVs. However, the compromised interaction between EVs and traditional interfaces limits their capture efficiency. The micro/nano-scale channels in microfluidic chips can enhance the contact frequency between recognition ligands on the chips and molecules on EV membranes, thus increasing EV capture efficiency. For example, the herringbone microfluidic chip, comprising patterned microgrooves, enhances fluid mixing efficiency by manipulating flow states and forming helical motions [33]. Therefore, collision between biological targets and affinity trapping substrates is improved, resulting in improved EV capture efficiency [34].

However, the near-surface hydrodynamic resistance decreases mass transfer in the microchannel [35]. To overcome this near-surface hydrodynamic resistance, Li et al. developed a 3D porous sponge microfluidic chip made by salt-crystallization, which provided a high surface-to-volume ratio [8]. Otherwise, researchers also developed a fluid nanoporous microinterface (FluidporeFace) in a herringbone microfluidic chip for efficient capture of tumor-derived EVs (Figure 2a) [34]. Supported lipid bilayers (SLBs) were encapsulated on the nanoporous herringbone microstructures, which not only improved the mass transfer, but also enabled multivalent recognition of aptamers, thus achieving multi-scale enhanced affinity reaction. As a result, the affinity increased by ~83-fold compared with the nonfluid interface. In addition, they designed a microfluidic chip to create a dynamic multivalent magnetic interface, enhancing the kinetics and thermodynamics of biomolecular recognition for the efficient isolation of EVs derived from tumors (T-EVs) [36]. Utilizing magnetic and flow fields, this engineered interface achieved a harmonious balance of affinity, selectivity, reversibility, and extendibility. As a result, they achieved a high-throughput recovery of T-EVs, facilitating comprehensive protein profiling. However, when utilizing a single ligand for EV capture and another ligand for EV detection, it is challenging to eliminate the interference of free proteins and obtain the requisite subtypes of EVs, thereby rendering it inadequate for clinical applications. To eliminate interference from free proteins, Zhang et al. designed a microfluidic differentiation method that accurately captured PD-L1⁺ EVs [37]. PD-L1⁺ EVs were labeled with biotin using DNA computation, incorporating dual input of lipid probes and PD-L1 aptamer. Subsequently, these labeled EVs were captured with streptavidin-modified microfluidic chips selectively.

Different subtypes of EVs represent distinct sources and diverse biological functions. Therefore, analysis of EV subtypes is crucial for studying the biological mechanism of EVs. On the contrary, all antibody-based enrichment systems are limited to highly specific isolation protocols, which result in partial EV loss due to differences in the expression of EV membrane surface proteins. Chen et al. proposed a novel herringbone microfluidics device, which not only possessed the advantages of herringbone microfluidics, but also incorporated aptamer-functionalized core-shell bar code (AFCSBs) [33]. Because their antiopal hydrogel shells have abundant interconnecting pores, barcodes can provide a rich surface area for anchoring of multiple DNA aptamers, enabling specific capture of multiple tumor-derived exosomes. However, its essence lies in using one type of aptamer for molecular capture and another type for molecular discrimination, making it still unable to distinguish different EVs. In order to isolate subtypes of EVs, MUN et al. developed a microfluidic chip-based magnetically labeled exosome isolation system (MEIS-chip) that involved magnetic nanoclusters (MNCs) conjugated with CD63 and HER2 with different degrees of magnetization (CD63 conjugated with low-saturation magnetized MNCs, CD63-LMC, and HER2 conjugated with high-saturation magnetized MNCs, HER2-HMC) [38]. Common exosomes were captured by CD63-LMC, while exosomes with HER-overexpression bound to both CD63-LMC and HER2-HMC simultaneously. This allows for the acquisition of varying degrees of magnetic particles and magnetic separation in the MEIS-chip via a magnetic field, ultimately resulting in the separation of different EV populations. Moreover, Chen et al. designed a nanoscale cytometry platform called NanoEPIC to enable the collection of small EVs (sEVs) bearing four different expression levels of PD-L1 by labeling them with antibody-functionalized magnetic nanoparticles (MNPs) (Figure 2b) [39]. EVs with higher PD-L1 expression levels had greater lateral deflection towards the edge of the device in the microfluidic flow channel due to their increased magnetic susceptibility resulting from binding to more MNPs. This facilitated the separation of sEVs based on four levels of PD-L1 expression: negative, low (exo-L), medium (exo-M), and high (exo-H). Lu et al. achieved the isolation of tumor PD-L1⁺ EVs and non-tumor PD-L1⁺ EVs through DNA logic-mediated double aptamer recognition and tandem chip for the first time (Figure 2c) [40]. Tumor-derived EVs were identified by EpCAM and PD-L1 nucleic acid ligands, inducing the "AND" logic operation, whereas non-tumor-derived PD-L1⁺ EVs only express PD-L1, thus invoking the "NOT" logic operation. These two independent outputs facilitated the separation of tumor and non-tumor origin PD-L1⁺ EVs through tandem microfluidics, respectively. Consequently, utilizing a streptavidin-functionalized microfluidic chip (T-Chip), only tumor-derived

PD-L1⁺ EV populations can be isolated. After excluding tumor-derived PD-L1⁺ EVs, the remaining PD-L1⁺ EVs from normal cells can be captured through hybridization between the extension sequence on the PD-L1 probe and the corresponding cDNA modified on the second microfluidic chip (N-Chip).

Currently, the microfluidic technology based on affinity separation has made tremendous advancements. There have been significant improvements in purification, capture efficiency, and subpopulation separation. Despite some progress in various studies, there are still certain limitations. To effectively apply these technologies to clinical diagnosis and precision treatment, further innovation and improvement are still needed. In short, the efficient isolation of EVs is the premise for researching their biological function research and clinical application. It is still necessary to develop new methods for the high-efficiency and high-purity isolation of EV subtypes.

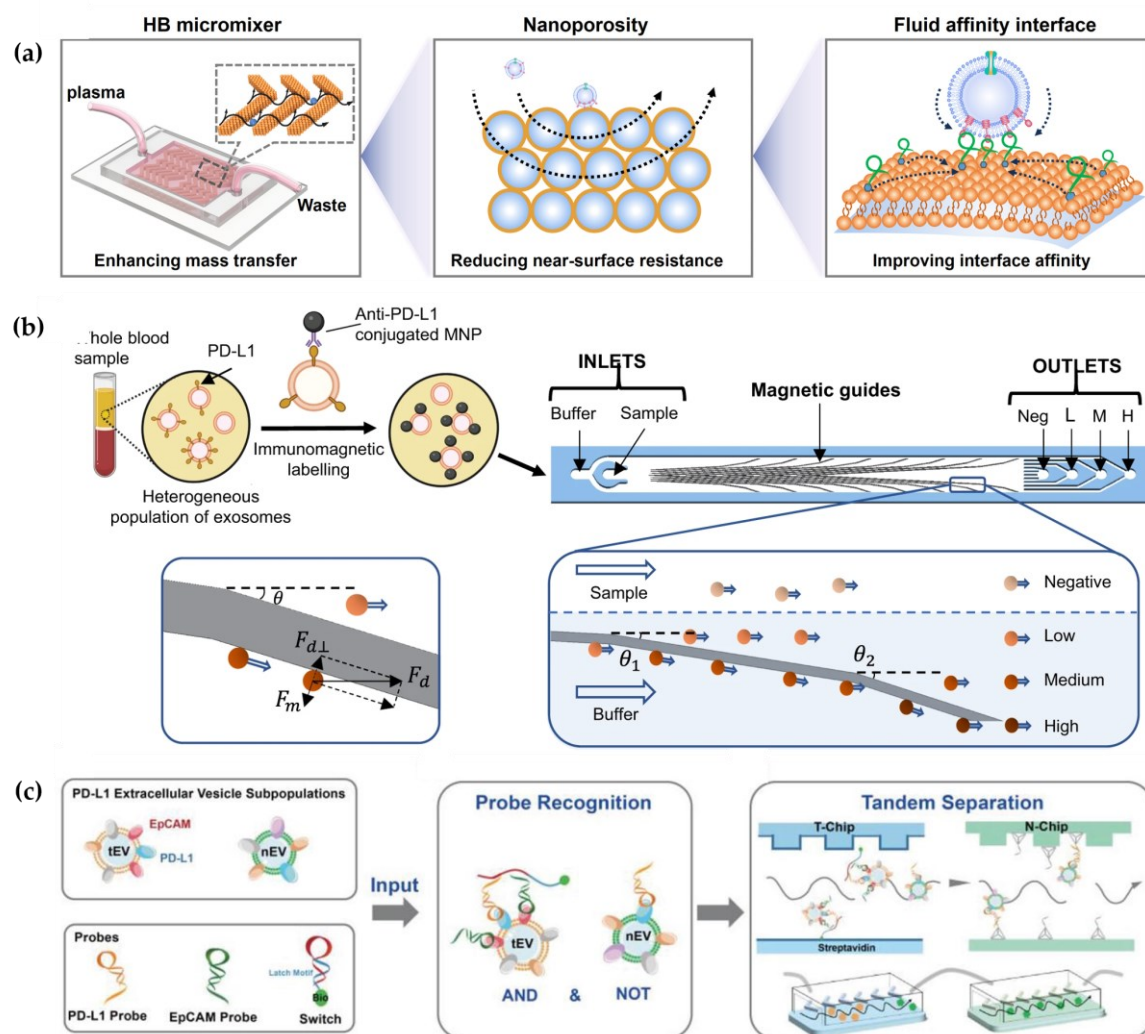


Figure 2. Affinity-based microfluidic EVs isolation methods. (a) The schematic diagram of FluidporeFace-Chip. Reproduced from Ref. [34]. (b) Magnetic deflection-based NanoEPIC system to achieve phenotypic profiling and nanoscale sorting of sEVs. Reproduced from Ref. [39]. (c) DNA computation mediated microfluidic tandem separation for PD-L1⁺ EVs Subpopulations. Reproduced from Ref. [40].

3. Microfluidic-based fluorescent detection.

3.1. Fluorescent detection

Fluorescence technology combined with microfluidic platform has been widely used in EV detection, which has the characteristics of fast response, good precision and high sensitivity. After

capturing EVs in microfluidic chips, membrane stains or fluorescent labeled antibodies are usually used to identify EVs [41]. For example, Kanwar et al. used a fluorescent carbanine dye (DiO) to stain exosome membrane, and counted the total number of exosomes captured on a microfluidic device (ExoChip) [42]. Antibodies against EV specific biomarkers, such as CD63, CD9 and CD81 are used for EV identification and quantification [43]. Besides, fluorescent labeled antibodies against disease related biomarkers on EV membrane are usually used for quantitative and qualitative analysis of EV biomarkers, thus reflecting the progression of diseases. For example, Hisey et al. captured ovarian cancer exosomes in a herringbone groove microfluidic device and quantified EpCAM⁺ exosomes [44]. The quantitative results showed that EpCAM⁺ exosomes were related with HGSOV disease progression. After utilizing the integrated microfluidic Exosome Separation and Detection System (EXID System), Lu et al. examined the abundance of exosomal PD-L1. [45]. Using the EXID system, a significant difference in fluorescence intensity was observed. The strategy had a limit of detection (LOD) of 10.76 / μ L and exosomal PD-L1 level reflected the sensibility for immune response. The conventional methods offer bulk information of proteins on EVs, which hardly enables absolute quantification. Liu et al. constructed “exosome-magnetic microbead-enzymatic reporter” complexes and encapsulated the complexes into droplets which ensured a single complex was encapsulated in a droplet. As a result, cancer-specific exosomes were absolute counted with a low limit of detection of 10 exosomes μ L⁻¹ (Figure 3a) [46].

The heterogeneity of EVs challenges the acknowledgement of their biological significance and clinical application. To explore the heterogeneity of EVs, Zhang's group developed a microfluidic chip featuring self-assembled 3D herringbone nanopatterns, enabling highly sensitive fluorescent detection of EV surface proteins [47]. The device was used to detect exosome subtypes expressing CD24, EpCAM, and FRalpha proteins in 2 μ L plasma samples from 20 ovarian cancer patients, and to suggest exosomal FRalpha as a promising biomarker in the early detection and monitoring of ovarian cancer progression. Furthermore, MMP14 on EVs holds potential for early detection and prognosis assessment in breast cancer metastasis [48]. The above microfluidic devices need cumbersome fabrication processes. Besides, the interaction between proteins and antibodies may be limited due to the steric hindrance caused by the posttranslational modification of proteins [49,50]. Sun's group developed a microfluidic thermophoresis device, which accumulated particles in a size-dependent manner and amplified fluorescence signals based on the different diffusion rates of particles in nonuniform temperature field [51]. Seven fluorescently labeled aptamers targeting different epitopes were employed for subtyping analysis of EVs. This method demonstrated a sensitivity and specificity of 95% and 100%, respectively, in cancer detection, and it can also be utilized for cancer classification. Furthermore, the method is applicable for the proteomic analysis of EVs in breast cancer, enabling the identification of metastatic breast cancer, monitoring treatment responses, and predicting patients' progression-free survival rates [52].

In addition to phenotypic heterogeneity, the tracing of EV origins is also particularly important. It can accurately detect and monitor the progression of diseases. Sun's group utilized microfluidic thermophoresis devices for the specific detection of tumor-derived EVs, achieving an accuracy of 97% [53]. Similarly, a rapid and non-invasive diagnostic assay, named the one-step thermophoretic AND gate operation (Tango), has been developed for precise identification of prostate cancer (PCa)-derived EVs directly in serum samples within 15 minutes. This method demonstrated an impressive overall accuracy of 91% in discerning PCa from benign prostatic hyperplasia (BPH) using a streamlined single-step format. This innovative technique holds tremendous potential for the swift and non-invasive diagnosis of cancers (Figure 3b) [54].

Besides proteins on EV surface, miRNAs are also important biomarkers and therapeutic targets for diseases [55]. Sun's group demonstrated a thermal swim sensor (TSN) employing nanoflakes for the in situ detection of exosomal miRNA, eliminating the need for RNA extraction or target amplification. Through the thermophoretic accumulation of nanoflake-treated exosomes, a heightened fluorescent signal was produced upon binding with exosome miRNA, facilitating the direct quantitative assessment of exosome miRNA. [56]. Afterwards, they devised a DNA tetrahedron-based thermophoretic analysis (DTTA) for the on-situ detection of mRNA in EVs,

achieving remarkable sensitivity and specificity [57]. Recently, they developed a DTTA for high sensitive and selective in situ detection of mature miRNA in EVs. This assay achieved a detection limit of 2.05 fM for mature miRNA in EVs without interference from pre-miRNA, and distinguishes between breast cancer patients and healthy donors with an overall accuracy of 90% (Figure 3c) [58].

Moreover, cell culture supernatant or EVs in body fluids lose the interaction information with other symbiotic cells in tissues, making it unable to accurately represent the role of EVs in intercellular communication [59]. Ji et al. employed spatially patterned antibody barcodes, achieved the multiplexed profiling of single-cell EV secretion from over 1,000 individual cells concurrently. This innovative approach enabled the comprehensive characterization of human oral squamous cell carcinoma, unraveling previously obscured single-cell heterogeneity in EV secretion dynamics (Figure 3d) [60]. This technology facilitates a thorough assessment of EV secretion diversity at the single-cell level, offering an invaluable tool to supplement existing single-cell analysis and EV research. Afterwards, they applied this platform to analyze the characteristic spectra of paired neuronal-microglial and neuronal-astrocyte single cells in the human cell lineage. These results provides a basis for exploring how neurons and immune cells interact through complex secretion networks [61].

While fluorescence technology utilizing microfluidic techniques has been successfully employed in EV detection, offering commendable accuracy and high sensitivity, it is not without its drawbacks. Some ons include the intricate preparation of fluorescence labels and the occurrence of spectral interference. This interference encompasses background interference and spectral overlap issues, along with the phenomenon of photobleaching in fluorescent molecules. The resolution of these issues will contribute to enhancing the accuracy of fluorescence detection of EVs.

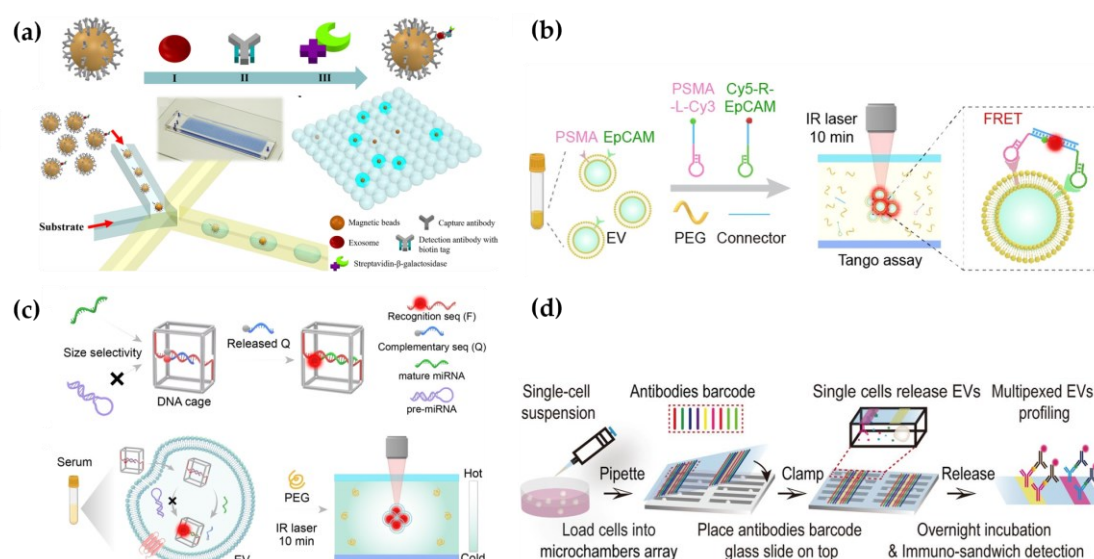


Figure 3. Microfluidic-based fluorescent detection. (a) The droplet digital ExoELISA method for quantifying EVs. Reproduced from Ref. [46]. (b) Schematic of the one-step thermophoretic AND gate operation (Tango) assay. Reproduced from Ref. [54]. (c) DTTA for detecting mRNA within EVs. Reproduced from Ref. [58]. (d) Schematic illustration depicting the workflow for the multiplexed profiling of single-cell EV secretion. Reproduced from Ref. [60].

3.2. Visualization detection.

In recent years, the method of detecting EVs through microfluidic technology using the colorimetric method has undergone significant development and has simplified the equipment [26,62,63]. Visualization detection relies on the change that can be observed by naked eyes, such as color changes within the detection system, which occur as a result of chemical or biochemical interactions between specific target analytes and colorimetric probes. One significant advantage of visualization assays is their independence from bulky off-chip detection systems. Consequently,

visualization detection has garnered growing interest in biomedical research, particularly for disease diagnosis, owing to its distinct advantages in EVs detection [59].

For instance, Chen et al. introduced a traditional colorimetric technique to detect EVs with a 3D scaffold chip [64]. They proposed a ZnO nanowire coated 3D scaffold chip device for effective immune capture and classical visual and colorimetric detection of EVs. In the work by Di et al., a rapid analysis method was introduced, utilizing nano-enzyme-assisted immunosorbent assay, eliminating the need for antibody detection [65]. The approach involved the immobilization of nanoparticles on the phospholipid membrane of exosomes, followed by the addition of chromogenic agents. For another, Jiang's group developed a sensor platform, which can be visually analyzed EV surface proteins in minutes. The sensor consists of a gold nanoparticle (AuNP) and a set of aptamers [66]. In addition, Ko et al. engineered a photofluidic platform powered by smartphones to quantify brain-derived exosomes. This innovative chip enables rapid processing, delivering results within one hour, which is ten-fold quicker than conventional methods. The device boasts a detection limit of approximately 10^7 exosomes/mL [67]. Utilizing enzyme amplification, it can detect exosome biomarkers, with results easily read through a smartphone camera.

Although many researchers have made incredible progress in the field of visualization detection methods, which can rapidly detect a wide range of biomolecules, from infectious diseases-related protein biomarkers to glucose and nucleic acids, the extensive application of visualization detection is limited. It is mainly used in underdeveloped regions, and low sensitivity is its major drawback.

3.3. Electrochemical detection

Electrochemical methods transfer the signals of EV recognition to electrochemical signals, such as voltage, current, and resistance [68]. Microfluidic-based electrochemical techniques have attracted great attention in EV detection due to their broad detection range, high sensitivity and specificity.

Affinity ligands are usually modified in microfluidic chips for EV capture. Then, the electrochemically responsive molecules were triggered to cause change of electrochemical signals. To date, great efforts have been devoted to introduce various signal production and amplification strategies, such as metal nanoparticles, tetrahedral DNA nanostructures (TDN), and nucleic acid-based amplification analysis to electrochemical biosensors for EV detection. For example, Wang et al. have developed a new filter-electrochemical microfluidic chip (FEMC) that integrated on-chip separation and in situ surface protein electrochemical analysis of exosomes in whole blood of breast cancer patients (Figure 4a) [69]. In this system, Zirconium-based metal-organic frameworks (Zr-MOFs) loaded with numerous electroactive methylene blue molecules (Zr-MOFMB@UiO-66) were attached to exosomes collected on electrode surfaces, leading to the amplification of electrical signals. The entire FEMC assay took 1 hour to complete, enabling timely and more informed opportunities for the diagnosis of breast cancer. To highly sensitively detect colorectal cancer exosomes, a microfluidic electrochemical biosensing platform based on TDN-based signal amplification was constructed [70]. TDN including EpCAM aptamer was immobilized on Au nanoparticles (AuNPs) as a recognition element to harvest the exosomes. Then, the AuNPs had an obvious catalytic effect on the redox reaction of ferricyanide, enabling electrochemical detection [Microfluidic Technology for the Isolation and Analysis of Exosomes]. The platform had a broad measurement range ($50\text{--}10^5$ particles/ μL) and a low limit of detection (42 particles/ μL).

Besides, an increasing number of nucleic acid amplification methods have been employed in electrochemical biosensors. Xu et al. proposed a two-stage microfluidic platform (ExoPCD-chip) for the electrochemical analysis of hepatocellular exosomes in serum (Figure 4b) [71]. Particularly, exosomes captured by electrochemical aptasensors with a CD63 aptamer led to the accumulation of the hemin/G-quadruplex. This complex could function as a NADH oxidase and horseradish peroxidase (HRP)-mimicking DNAzyme simultaneously. Thus, the freshly formed H_2O_2 by NADH oxidation could be continuously catalyzed, accompanied by significant signal enhancement [72]. Moreover, a staggered Y-shaped micropillar mixing pattern was introduced to create an anisotropic flow without any surface modification to improve exosome enrichment efficiency. Due to the flexible programmability, aptamers are easily engineered for signal amplification, to improve EV detection

sensitivity. For example, a hemin/G-quadruplex system and rolling circle amplification (RCA) were combined in an aptasensor for the selective and sensitive detection of gastric cancer exosomes (Figure 4c) [73]. RCA is recognized as a nucleic acid amplification analysis that can be performed at room temperature to preserve the integrity of exosomes. In addition, Zhang et al. designed a remarkably selective electrochemical micro-aptasensor with a detection limit of 5×10^2 exosomes/mL by integrating a micropatterned electrochemical aptasensor and a signal amplification strategy of hybridization chain reaction (HCR) (Figure 4d) [74]. Biotin-labeled HCR products were used to bind specifically to enriched exosomes utilizing EpCAM aptamers as a bridge. This was followed by the attachment of multiple avidin-HRPs, producing a current signal through the enzyme reaction. Moreover, the proposed aptasensor was effective in discriminating serum samples from early-stage lung cancer patients and late-stage patients, indicating significant promise for early cancer diagnosis.

To sum up, electrochemistry proves highly suitable for EV analysis within an integrated microfluidic chip, offering a multitude of advantages. Furthermore, no requirements for optical transparency expand the choice of materials in electrochemical response. However, contamination and changes in pH, temperature, and ionic concentration often influence the lifetimes of electrodes, which needs to be addressed [75]. So far, only a limited number of microfluidic devices incorporated with electrochemical techniques have been developed for EV detection. We believe that there will be a growing number of microfluidic devices combined with electrochemical detection as a promising means for point-of-care diagnostics.

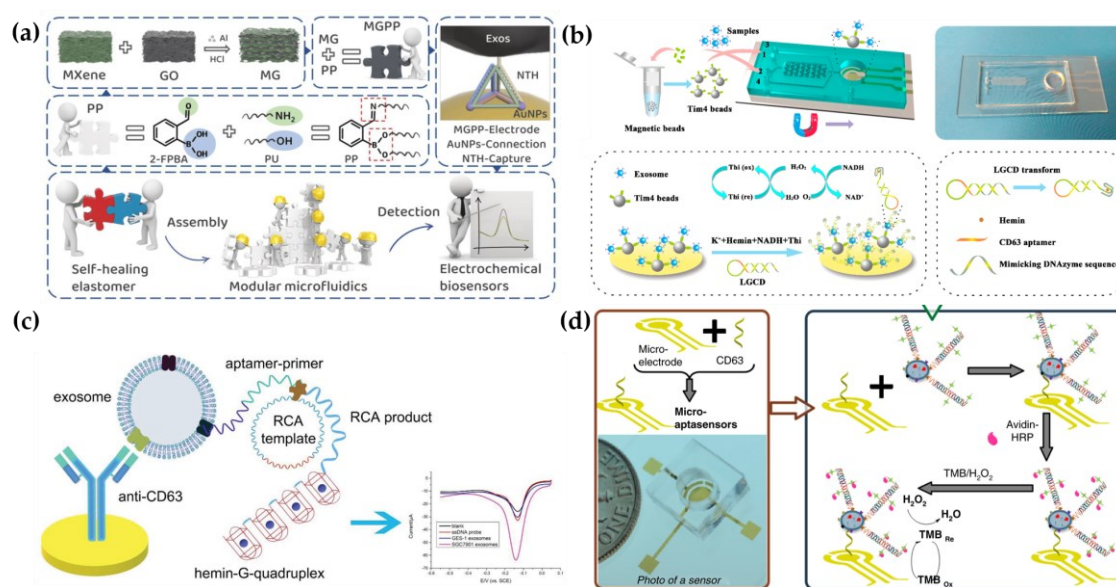


Figure 4. Microfluidic-based electrochemical detection. (a) Filter-electrochemical microfluidic chip (FEMC) for exosome detection. Reproduced from Ref. [69]. (b) Schematic diagram of the ExoPCD-chip. Reproduced from Ref. [71]. (c) Hemin/G-Quadruplex-Assisted signal amplification. Reproduced from Ref. [73]. (d) Electrochemical micro-aptasensors based on HCR for exosome detection. Reproduced from Ref. [74].

3.4. Surface enhanced Raman scattering (SERS)

Surface enhanced Raman spectroscopy (SERS) effectively generates spectra on certain metal surfaces, providing vibrational and rotational energy information of molecules, which is reflected in spectral peaks used to specifically identify molecules [75,76]. However, SERS measurements present significant challenges in reproducibility and sensitivity [76]. In this aspect, microfluidic surface-enhanced Raman spectroscopy (MF-SERS) is making progress in resolving some significant and previously insurmountable issues and limitations of SERS detection to some extent, thus improving detection capability and extending its application [78]. Through the utilization of high-throughput

nanosurface microfluidics control technology and unique fingerprint identification, precise testing of ultra-small populations of biochemical particles, such as cancer EVs.

In efforts to amplify Raman signals from cancer-derived EVs, Mahsa et al. developed a nanosurface fluidic device for label-free, non-immunological SERS detection of EVs. This device effectively distinguished the SERS fingerprint of EVs from noncancerous glial cells (NHA) and two sub-populations of the GBM EVs (i.e. U87 and U373). The sample solution flowed from the input ports to the serpentine analysis channels ($50 \times 250 \mu\text{m}^2$) to achieve a single-layer distribution of EVs on the nanosurface. At the same time, metal nanomaterials with SERS activity formed a hexagonal nanoscale triangular array, with each two triangles forming a bowtie structure with a suspended gap region area to amplify the EM-field enhancement, electromagnetic field enhancement factor of 9×10^5 (Figure 5a) [79]. In addition to the above-mentioned label-free microfluidic Raman chip, Wang et al. developed a new one with immunoassay for quickly and sensitively detecting the exosomes (Figure 3b) [80]. Hybrid channels of triangular column arrays were used to enrich CD63-positive exosomes and fixed them in the Raman detection region. EpCAM-labeled Raman beads with high densities of nitrile were used as probes for detection, and the detection limit was 1.6×10^2 particles per mL with 20 μL samples.

Various biomarkers can indicate diverse biological functions, making it significant to detect multiple exogenous biomarkers. Han et al. proposed a microfluidic-SERS technique for profiling numerous exosomal biomarkers to diagnose osteosarcoma. Gold nanoparticles labeled with SERS tags can selectively bind to exosomes using specific antibodies in samples, forming exosome immunocomplexes. A microfluidic chip, comprising two symmetrical polydimethylsiloxane (PDMS) layers and a nanoporous polycarbonate track-etched (PCTE) membrane, was employed for exosome purification [81]. Microfluidic tangential flow filtration effectively eliminated plasma biomolecules and free SERS tags, while enriching exosome immunocomplexes on the membrane for in situ SERS analysis [82]. Herein, Wang et al. also showcased a multiplex EV phenotype analyzer chip (EPAC). EPAC integrates a nanomixing enhanced microchip and a multiplex surface-enhanced Raman scattering (SERS) nanotag system for direct EV phenotyping. [62,81]. They observed the EV phenotypic heterogeneity and longitudinally monitored the EV phenotypic evolution, finding specific EV profiles involved in the development of drug resistance and the potential of EV phenotyping for monitoring treatment responses [83]. (Figure 5c) Thus, the microfluidic-SERS method offers great potential for the detection of external vesicles and cancer diagnosis.

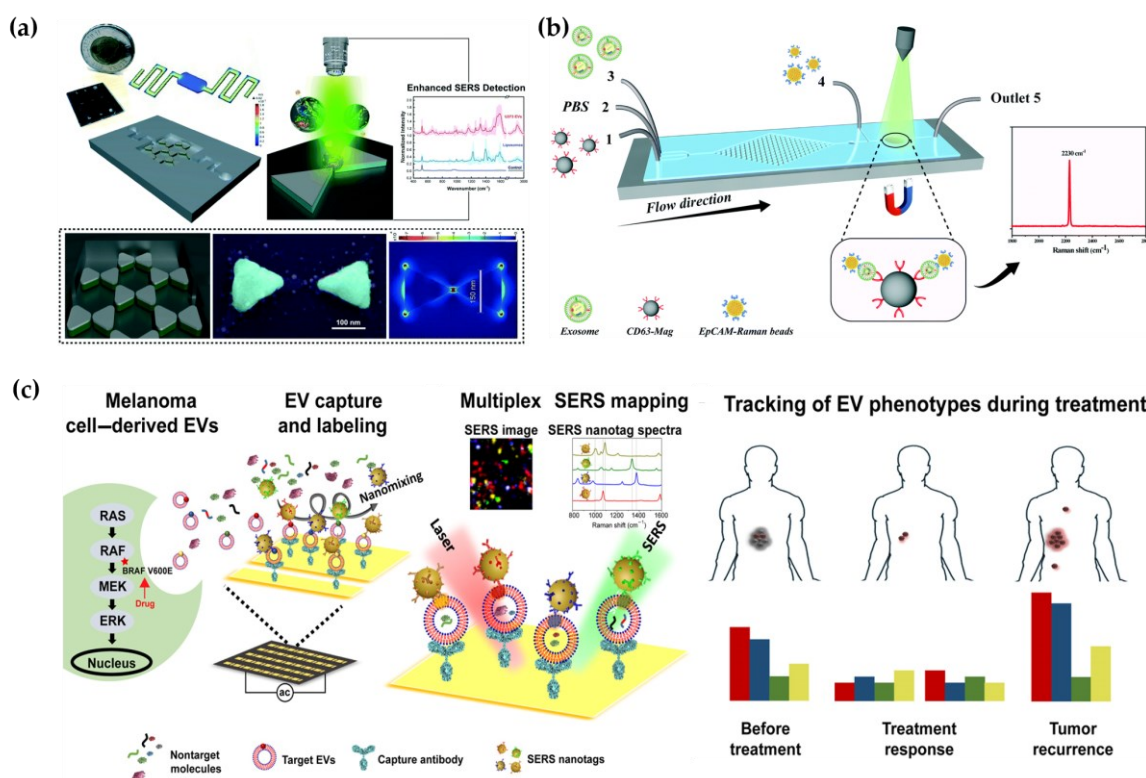


Figure 5. Microfluidic-based SERS detection. (a) Spatially suspended nanobowtie surface microfluidic device for SERS detection of EVs. Reproduced from Ref. [78]. (b) Microfluidic Raman chip for exosome capture and detection. Reproduced from Ref. [79]. (c) EV phenotyping by EPAC. Reproduced from Ref. [82].

4. Conclusions and Perspectives

EVs are intricately linked to numerous physiological processes as well as the onset and progression of diseases. Efficient EV isolation methods and sensitive EV detection methods will help to improve the understanding of the physiological and pathological effects of EVs, and provide important support for the precision medicine of related diseases. At present, the conventional EV isolation and detection technologies still have limitations. Microfluidic-based methods for isolation and detection of EVs have obvious advantages of high integration, low consumption, fast speed, high separation efficient and high detection sensitivity, which opens up new ideas and directions for the research of EVs. With the help of microfluidic chips, the efficient isolation, enrichment and multi-marker detection of EVs with different sizes can be integrated into a single chip, and a more diversified clinical detection instrument can be built.

The field of microfluidic-based EV isolation and detection is still in its infancy, and there are still a lot of theoretical and technical problems to be solved. Therefore, the means to achieve highly selective and accurate isolation of EVs in actual biological samples, achieve sensitive and selective detection of these EVs and even the biological information carried in them will be important topics in the study of EVs based on microfluidic chips. With the development of technology and in-depth research, the isolation and analysis of individual EVs can be realized by microfluidic method, and commercial EV chips are also expected to be applied in clinical practice.

In this article, the existing methods of microfluidic-based EV isolation and analysis are reviewed. Compared with traditional ultracentrifugation, ultrafiltration, immunocapture and co-precipitation, microfluidic chips are smaller and more flexible, and microfluidic immunoaffinity methods can isolate high-purity EVs with strong specificity. The isolation methods based on the physical characteristics of EVs do not need to add expensive reagents such as antigens and antibodies. Therefore, the cost is low, and the isolation process will not cause contamination, which is conducive

to downstream analysis. Microfluidic-based EV analysis methods have the advantages of fast analysis speed, high throughput and low reagent consumption, which can meet the needs of rapid detection of EVs in a large number of clinical samples. Therefore, microfluidic technology has significant advantages in the isolation of EVs in a small amount of clinical samples and the rapid estimation of diseases.

Although microfluidic technology has made remarkable progress in the field of EV isolation and analysis. It usually needs to be combined with some instruments such as injection pumps and fluorescence detectors, which limits its application scenarios to a certain extent. In addition, EVs are small in size and similar in density to body fluids, a single separation method is inadequate. How to balance the relationship between EV purity and recovery on microfluidic chips is also a great challenge.

In recent years, with the rapid development of micro/nano manufacture, new materials and information technology, the design of microfluidic chips and the performance of supporting devices will be further improved. It is mainly reflected in: (1) The development of precision manufacture technology makes it possible to integrate multiple EV isolation methods and realize the integration of EV isolation and detection on one chip; (2) By combining the chips and portable detection equipments, a miniaturized EV microfluidic isolation and analysis platform is constructed to realize the rapid detection of EVs and greatly expand its application space. With the miniaturization, integration and automation of microfluidic EV isolation and analysis devices, microfluidic technology will play an increasingly important role in EV isolation, biochemical detection and mechanism research.

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