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The new advance of exosome-based liquid biopsy for cancer diagnosis

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Abstract Liquid biopsy is a minimally invasive method that uses biofluid samples instead of tissue samples for cancer diagnosis. Exosomes are small extracellular vesicles secreted by donor cells and act as mediators of intercellular communication in human health and disease. Due to their important roles, exosomes have been considered as promising biomarkers for liquid biopsy. However, traditional methods for exosome isolation and cargo detection methods are time-consuming and inefficient, limiting their practical application. In the past decades, many new strategies, such as microfluidic chips, nanowire arrays and electrochemical biosensors, have been proposed to achieve rapid, accurate and high-throughput detection and analysis of exosomes. In this review, we discussed about the new advance in exosome-based liquid biopsy technology, including isolation, enrichment, cargo detection and analysis approaches. The comparison of currently available methods is also included. Finally, we summarized the advantages and limitations of the present strategies and further gave a perspective to their future translational use.

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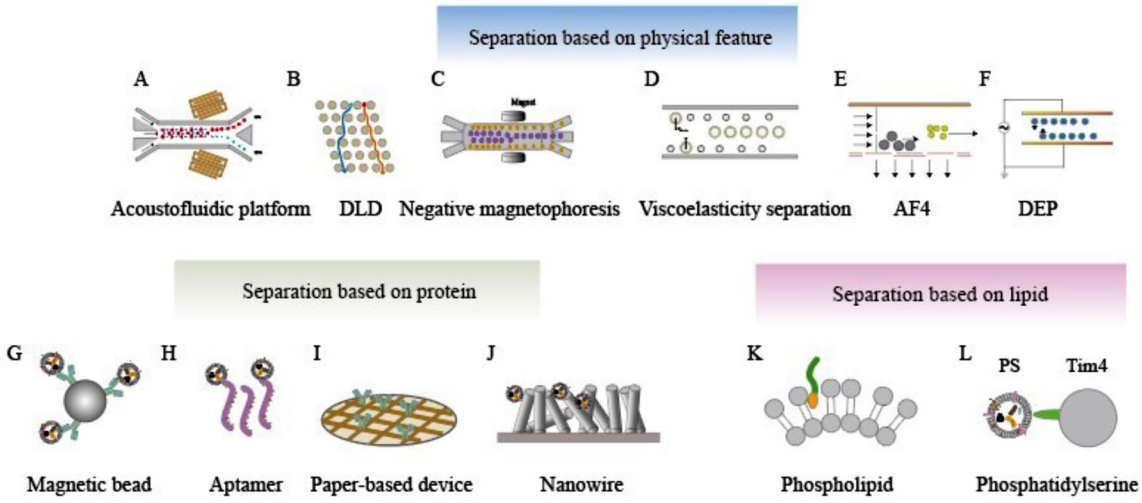
Graphical Abstract



Keywords Exosomes, Liquid biopsy, Cancer, Diagnosis, Biomarker

Introduction

Cancer is a leading cause of death worldwide [1]. Tissue biopsy has been considered as the standard method for cancer diagnosis [2]. However, this invasive method may cause potential risks such as cancer metastasis and infection. In addition, due to the heterogeneity of tumors, tissue biopsy can only reflect the pathological status of a certain location, but is hard to reflect the overall status of tumors. Liquid biopsy refers to a technique which diagnoses diseases by detecting circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and exosomes from cerebrospinal fluid, saliva, pleural fluid, blood, ascites, urine and other body fluids [3]. Compared with tissue biopsy, liquid biopsy mainly has the advantages of minimal invasion, early diagnosis and dynamic monitoring. At present, the detection of CTCs and ctDNA is of potential significance in the dynamic monitoring of tumor progression, metastasis and drug resistance [4–8]. However, their clinical application still faces many challenges. First, although ctDNA can reflect the mutation of tumor cells, current detection method will be unreliable when variant allele frequency (VAF) is below 0.5% [9]. Second, the abundance of ctDNA is unstable since its half-lifeis generally less than two hours [10]. Third, due to the low abundance and heterogeneity of CTCs in blood, the enrichment and detection is challenging, which restrains its clinical application [11].

Exosomes are vesicles with lipid bilayer membrane ranging from 40 to 160 nm and contain abundant bioactive molecules such as proteins, DNA, mRNA and non-coding RNAs (including lncRNA, miRNA, circRNA and piRNA) [12, 13]. Compared with CTC and ctDNA, exosome-based liquid biopsy exhibits unique superiority. Exosomes carry a variety of cargoes from donor cells, both protein and nucleic acid can provide more comprehensive information. Moreover, exosomes are more abundant than CTC and ctDNA, making it easier to be enriched from clinical samples. In addition, exosomes with lipid bilayer structure effectively protect cargos from degradation, which ensures the reliability for downstream analysis [14, 15]. However, the practical application of exosome-based liquid biopsy is limited by the lack of separation methods with high efficiency and purity. Also, specific and accurate detection methods which can distinguish tumor-derived exosomes to exclude the interference generated by exosomes from normal cells remain to be further investigated. Thus, the development of high-efficient and accurate technologies for exosomal separation and detection profoundly impacts the application of liquid biopsy [16–18]. In this review, we summarized the up-to-date exosomal isolation and cargo analysis technologies, as well as the clinical application of exosome-based liquid biopsy to provide frontier information in oncology diagnostic fields.

Biofunction and heterogeneity of exosomes

In recent years, an increasing number of researches have shown that exosome-mediated cellular communication is critical for cancer progression [19]. The function of exosomes is mainly achieved through the uptake by receptor

cells. However, the theory for uptake remains to be clarified. Generally, two theories have been proposed: clathrin or raft mediated endocytosis and direct fusion [20–22]. Once internalized, various cargoes, such as proteins, lipids, RNAs and DNAs, perform their biological functions by different mechanisms. For example, it has been demonstrated that clathrin light chain A (CLTA) derived from hepatocellular carcinoma (HCC) small extracellular vesicles (sEVs) had a role in the facilitation of microvascular niche development. This was achieved by the upregulation of basigin (BSG), which subsequently accelerated the process of metastasis [23]. Furthermore, it has been observed that exosomes derived from glioma cells were capable of transferring suprabasin (SBSN) to enhance the aggressiveness and progression of cancer. This effect was achieved by the activation of the NF-κB signaling pathway and NEMO ubiquitination [24]. In addition, colorectal cancer-derived miR-21-5p and miR-200a in sEV could polarize macrophages, inducing $CD8^+$T cell immunosuppression by increasing PD-L1 expression in tumor associated macrophages [19]. Exosomes from non-small cell lung cancer (NSCLC) cells containing lncRNA-SOX2OT promoted bone metastasis by regulating abnormal differentiation and dysfunction of osteoclasts [25]. Circ-CABIN1 from Glioblastoma-derived exosomes promoted temozolomide resistance by regulating olfactomedin-like 3 (OLFML3) [26].

However, exosome cargoes are not static, they differ from different cells, even the same cell, during different stages. The heterogeneity of exosomes reflects the state of donor cells, which are easily influenced by the microenvironment and inherent biology [27, 28]. Since exosomes mediate intercellular communication in multiple diseases, the heterogeneity of exosomes often determines their functional characteristics and the way they affect different aspects of cell biology. For instance, proteomic assessment of breast cancer-derived exosomes showed heterogeneity in donor cells' epithelial/mesenchymal phenotype, which indicated different cancer progression stages [29]. Due to the heterogeneity of exosomes and their important role in organisms, the development of more efficient isolation technology and accurate detection methods for contents are essential for disease diagnosis.

Isolation and enrichment technology of exosomes

Ultracentrifugation has been widely regarded as a common method for isolating exosomes [30]. This technique, often combined with ultrafiltration, allows for the separation of exosomes from plasma, serum or cell culture medium. However, the separation process involve multiple centrifugation steps, which are time-consuming and inefficient, potentially resulting in decreased exosome purity. Another commonly used method for exosome isolation is polymer-based precipitation technology. This approach typically involves mixing samples with a precipitation solution containing polymers such as polyethylene glycol (PEG), followed by incubation at 4 °C and subsequent low-speed centrifugation [31]. Compared with ultracentrifugation, precipitation method has low impact on isolated exosomes, but it may isolate non-vesicular contaminants (including lipoproteins) at the same time, and the mixing of polymer materials may affect the downstream analysis [32]. Moreover, classical separation and enrichment methods show their limitations when it comes to high throughput treatment since automation can hardly be achieved by conventional methods.

Promoting biomedicine and materials science has laid a solid foundation for novel technology development. Many exosomal separation systems, such as microfluidic devices and automation technology, have sprung up in the recent years. Here, we summarized up-to-date separation and enrichment methods according to different principles.

Separation and enrichment based on physical feature

Acoustofluidic platform

Acoustofluidics refers to the combination of acoustics and microfluidics (Fig. 1A). Controlled by acoustic radiation force and Stokes drag force, particles in channels can be separated based on their size and density [33]. In contrast to conventional isolation technology, the acoustofluidic platform does not need large-scale instrument, practically offering possibility to clinic application. Chen et al. developed the EXODUS system for exosome separation. In their research, with the help of high-frequency harmonic oscillations and low-frequency harmonic oscillations, the acoustofluidic streaming separated exosomes and limited particle aggregations. By applying dual-frequency transverse waves on a dual-membrane filter, the system realized rapid separation and purification of exosomes from biofluids [34]. In addition, Wang et al. developed an acoustofluidic device to separate salivary exosomes efficiently. Two separation modules were applied to isolate micrometer and submicrometer particles by using 20-MHz and 40-MHz surface acoustic waves (SAWs) respectively. The separated exosomes could be analyzed downstream for oropharyngeal cancer detection [35]. Yang et al. offered a new strategy to separating nanoparticles at a recovery efficiency of 92.6%. Based on a fan-shaped ultrahigh-frequency bulk acoustic waves (BAWs) resonator, the device could separate exosomes and microvesicles directly from human plasma [36].

However, acoustic radiation force alone is not fully enough to control small size particles. Several researches focused on integrating acoustofluidic platform with other methods

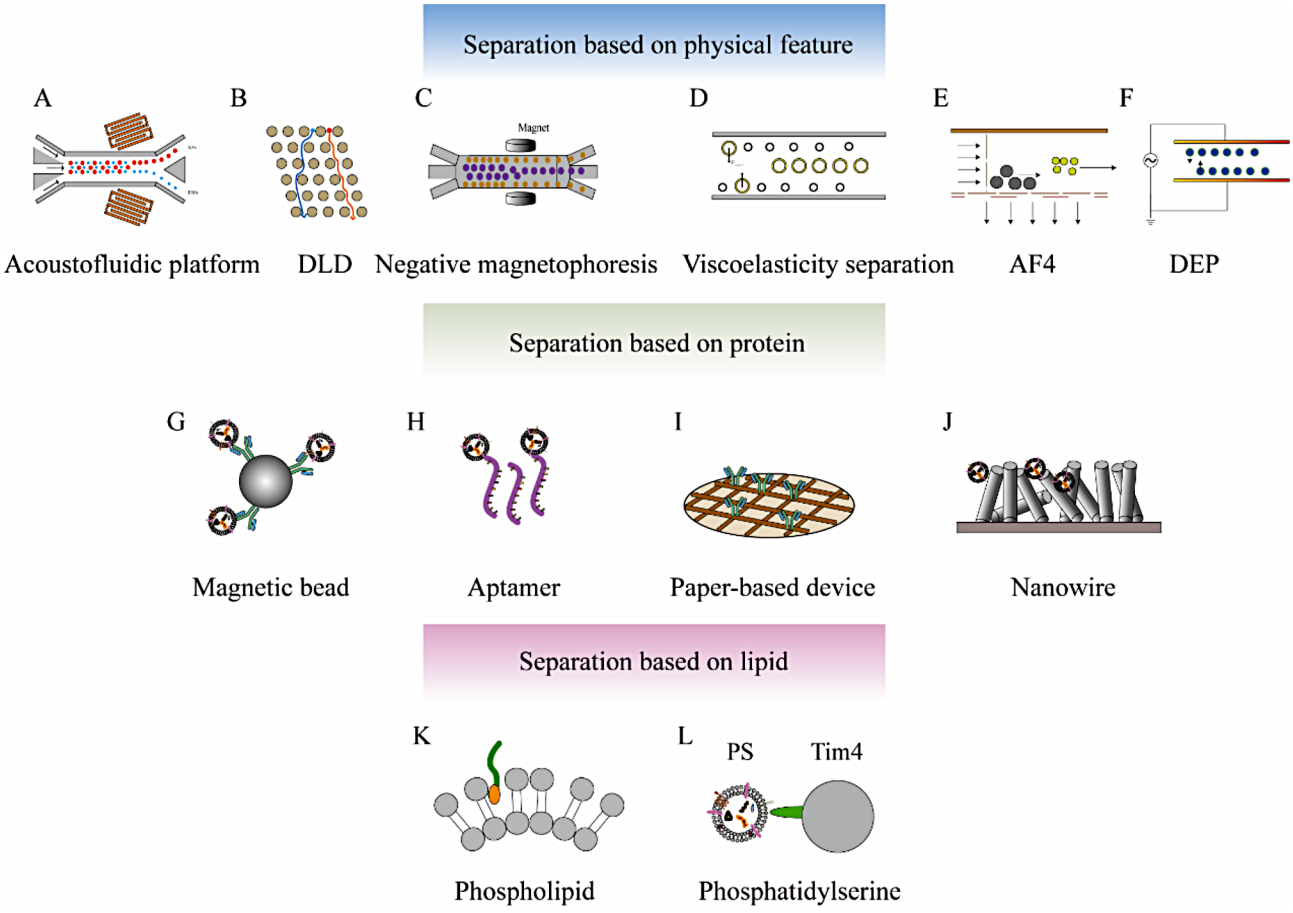


Fig. 1 New isolation and enrichment methods for exosomes. **A**. Separation based on acoustofluidic platform. **B**. Separation based on deterministic lateral displacement. **C**. Separation based on negative magnetophoretic technique. **D**. Viscoelasticity-based separation. **E**. Separation based on asymmetric-flow field-flow fractionation. **F**. Dielectrophoretic separation. **G**. Separation by magnetic beads modified with antibodies. **H**. Separation based on aptamer targeting surface proteins. **I**. Separation based on paper-based device. **J**. Nanowire-array-based separation. **K**. Phospholipid-based separation. **L**. Separation based on Tim4 beads targeting PS

to improve its performance. Dielectrophoretic force was combined with acoustophoretic force to separate particles bellow 200 nm. The device could achieve a purification efficiency of over 95% for exosomes separation [37]. Additionally, Dolatmoradi et al. proposed a thermo-acoustofluidic separation strategy to separate exosomes with higher than 93% efficiency. The difference of cholesterol content led to changes in acoustic contrast temperature (TΦ). Based on this, separation of vesicles was realized by adjusting temperature in the present of acoustophoresis [38]. A novel method named acoustofluidic centrifuge was creatively proposed by researchers. Separation and enrichment of nanoparticles was realized by the combination of acoustic streaming and droplet spinning. When SAWs propagated into the droplet located on the polydimethylsiloxane ring, the droplet spun, gathering particles together towards the center. Only 20μL sample was enough to separate particles below 100 nm in one minute [39]. Similarly, Dumčius et al. improved the acoustofluidic centrifuge system with a dual-wave mode which generated both Rayleigh and shear-horizontal SAW, making it possible to isolate particles at the size of less than 20 nm within 105 s [40]. It can be predicted that the combined use of multiple technologies will become the trend of future development in the nanoparticle separation area.

Deterministic lateral displacement (DLD)

The concept of deterministic lateral displacement (DLD) was initially introduced by Huang et al. in 2004. This technique enables the separation of various particles within a mixture by using their size differences (Fig. 1B). When laminar fluid is introduced into the chip, an array of micrometer-scale barriers positioned on the chip causes the fluid to bifurcate, particles that are smaller than the width of the lanes will follow the streamlines, while larger particles will be segregated into a different pathway [41]. Wunsch et al. designed a nanoscale lateral displacement array for effective separation of particles down to 20 nm [42]. Nevertheless, the device's functionality was limited to particle separation, as it did not possess the capability for quantitative determination.

To overcome the limitation of traditional DLD device, Zeming et al. designed a system which combined DLD and polymer microbeads, realizing DLD separation and quantitative detection at the same time. It is important to emphasize that the system could detect not just vesicles, but also DNA and proteins smaller than 50 nm. Regarding mechanism, bioparticles larger than 50 nm were captured by beads conjugated with antibodies, contributing to an increase in size. In contrast, bioparticles smaller than 50 nm, for example, human serum albumin, were bind to beads, resulting in electrostatic changes. The modulation of charge or size in microbeads would change their lateral displacement, the extent of which was related to the number of bioparticles, thus offering a sensitive way for separation and quantitative detection [43]. Although DLD has been proved to be a high-resolution, general-purpose particle separation method, some limitations remain, such as low throughput, high cost and complex device. System optimization and cost reduction may be the priority direction for future development.

Other label-free separation based on microfluidic systems

Negative magnetophoretic technique refers to a label-free method to separate particles with less magnetization than surrounding magnetic liquid (Fig. 1C). When exposed to magnetic fields, particles are manipulated by magnetic buoyancy force which is related to their volume. Big particles gather together in the center of channel due to larger forces, thus realizing size-based separation of different components. Zeng et al. developed a negative magnetophoretic separation method to promote the convenience of exosome extraction. The device employed a microfluidic chip, which included a magnetic pole channel, a separation channel, and two inlets. Using a biocompatible ferrofluid, the device effectively separated exosomes from the conditional medium of human fetal bone marrow mesenchymal stem cells at a purity of 80% [44]. Similarly, Xue et al. combined negative magnetophoresis and oscillatory flow in one microfluidic chip to extend trajectory of each particle in channel, thus realizing more sufficient separation for microparticles [45].

Viscoelasticity-based separation is another label-free way to separate exosomes (Fig. 1D). The system is simple and easy to operate since no other complicated units, such as magnetic poles, electric and acoustic fields, are needed. Samples mixed with poly-oxyethylene (PEO) solution were injected into channels along with guide fluids. When moving along the channel, particles in viscoelastic medium were subjected to elastic force, viscous drag, and inertial lift forces, all of which are related to particle diameter. Size-dependent separation was realized based on volumetric flow rates of both samples and guide flows [46]. Liu et al. established a device which isolated exosomes from lager extracellular vesicles by microfluidic viscoelastic flows. The addition of PEO solution generated elastic lift forces on vesicles. Large vesicles finally migrated to the centerline of flow while small vesicles, such as exosomes gathered together on two sides. The system was proved to separate exosomes at more than 90% purity [47]. Zhou et al. improved the viscoelastic separation system, replacing the straight channel with a reverse wavy channel structure to facilitate particle focusing. They separated exosomes with a purity higher than 92% [48]. Similarly, Bai et al. designed a dean-flow-coupled elasto-inertial microfluidic chip (DEIC) to realize exosome isolation from cell culture medium or human serum with low protein contaminants. Compared with straight channel, the spiral microchannel structure used by the system exhibited 3.4 fold improvement in focusing efficiency [49].

Asymmetric-flow field-flow fractionation (AF4) technology has been widely used for continuous particle separation (Fig. 1E). Briefly, the process could be divided into two stages; focus stage and elution stage. During the focus stage, two opposite flows limited samples into a thin band, gathering particles of different sizes together. Then, in the elution stage, by applying a single-direction flow, particles would be separated based on their hydrodynamic size and diffusion coefficient [50, 51]. However, AF4 separation method can only be used on the size-dependent level, isolation of particles with the same size but with different components such as surface proteins or other molecules is not supported.

Dielectrophoretic separation(DEP)refers to a continuous isolation strategy based on dielectrophoretic force, the magnitude of the force is related to dielectric properties of the particle and medium, particle size and electric field frequency (Fig. 1F). Particles show dielectrophoretic activity in the presence of a heterogeneous electric field, by adjusting electric field frequency, isolation of particles with different sizes can be realized. Lewis et al. introduced an efficient separation system for pancreatic ductal adenocarcinoma (PDAC) patient exosomes by combining a microarray chip with an alternating current electrokinetic field, which could directly separate exosomes from blood samples without sample pretreatment. Immunofluorescence analysis was subsequently applied to distinguish PDAC patients with healthy controls with 99% sensitivity [52]. Additionally, DEP has been combined with other technology, such as plasmonic sensing. Kwak et al. developed the KeyPLEX system to concentrate exosomes on plasmonic sensing surface functionlized with specific capture antibodies such as CD63, CD24 and EPCAM. By applying electroosmosis and dielectrophoretic forces, the system realized rapid isolation and

detection for ovarian cancer-derived exosomes in plasma within 10 min [53]. Since cancerous exosomes share similar physical features with those from normal cells, specific antibody or aptamer targeting tumor markers may be indispensable to reduce nonspecific binding.

Separation and enrichment based on membrane proteins

Magnetic separation

Magnetic separation is generally conducted by magnetic beads attached with peptides or antibodies targeting specific exosome biomarkers (Fig. 1G). Bathini et al. presented a novel liquid biopsy chip technology based on magnetic particles with synthetic peptides. By binding to the heat shock proteins (HSPs) on exosome surface, the magnetic bead could capture exosomes directly from conditioned media in 20 min [54]. Zhang et al. developed an automated exosome separation system named EVrich. By adding magnetic beads that combined CD9, one of the exosome markers on the surface, the system could realize high-throughput isolation of exosomes from urine samples after mix, incubation, washing and elution four steps. Compared with classic exosome separation methods such as ultracentrifugation, exosomes isolated by EVrich exhibited a more complete form, along with stable expression of characteristic proteins [55]. However, the device could not realize exosome isolation and quantitative determination at the same time. To overcome the limitation, Yu et al. constructed magnetic nanoparticles modified by EphA2 antibody (Z-aE2) and gold nanoparticles (Au-aG1) modified with GPC1 antibody (Au-aG1) to separate and quantitatively detect pancreatic cancer exosomes from patient serum, the concentration of $GPC1^+$ exosomes was positively related to Au level, thus realizing sensitive and quantitative determination [56]. Moreover, a single molecule array technology was developed to detect exosomes from colorectal cancer. With the aim of excluding interference from non-cancer-derived exosomes, two pairs of antibodies were used for exosome capture and detection. Magnetic beads modified with CD9 or EPCAM antibodies were used to separate total exosomes and cancer-derived exosomes respectively, while a biotinylated antibody for CD63 was used for generating fluorescent signals. By using TNM stage Multivariate Cox modelling, CD9-CD63 level was applied as an independent prognostic covariate for progression free survival ($p=0.048$) and overall survival ($p=0.0038$), Epcam-CD63 level functioned as an independent prognostic factor for overall survival ($p=0.01$), which indicated the clinical meaning of exosomal markers [57].

Due to the heterogeneity of exosomes, detection of exosomes by single protein biomarker cannot fully reflect the disease status. In order to overcome the limitation, a multi-color-emissive magneto-luminescent nanoarchitecture system was developed to capture Tenascin C (TNC), amphiregulin (AREG) and programmed cell death ligand-1 (PD-L1) positive exosomes simultaneously from whole blood sample. Antibodies conjugated with different color-emissive carbon dots were attached on magnetic nanoparticles to bind to three different exosomal proteins. After being separated by magnet, excitation light was conducted to image cancer-derived exosomes [58]. The platform has the potential to be utilized for cancer identification and prognostic evaluation, especially for metastasis, chemotherapy and immunotherapy resistance.

Classical immunomagnetic beads separation is limited to nonspecific interference of proteins or cell fragments. In order to exclude these contaminants, immunomagnetic hedgehog particles modified with anti-CD63 antibodies were used to capture exosomes with a 91.70% efficiency. Duo to the topological structures, only exosomes could be captured on particles with unique nanospikes. Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was subsequently used to release exosomes captured by magnetic beads for downstream analysis [59].

Aptamer-based separation

Aptamers are structured oligonucleotide sequences that can bind to target molecules with high specificity and affinity (Fig. 1H) [60]. Chen et al. designed a CD63-aptamer-modified polymorphic carbon (CoMPC@Au-Apt) to separate urinary exosomes from gastric cancer patients [61]. Niu et al. developed a fluid nanoporous microinterface (FluidporeFace) decorated with EpCAM aptamer modified liposomes on a microfluidic chip for tumor exosome separation. Notably, FluidporeFace used nanoporous herringbone structure to obtain enhanced mass transfer. Also, the fluid supported lipid bilayers (SLB) fabricated with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were chosen to achieve high fluidity and affinity. Compared with the conventional aptamer affinity interface, DOPC SLBs showed 83-fold higher in affinity, realizing efficient separation for tumor exosomes in a variety of body fluids [62].

In order to enhance the specificity for isolating tumor-derived exosomes, strategies based on dual-aptamer have been developed. Chen et al. constructed an ultra-sensitive assay that could accurately identify tumor-derived exosomes from a large number of normal cell-derived exosomes in blood samples from cancer patients. Based on EpCAM and CD63 dual-aptamer recognition technology, the system separated and detected cancer-derived exosomes with a 1/10,000 discrimination capability. The relative abundance between cancer exosomes and total exosomes calculated by fluorescence signals presented a clearly positive relationship with breast cancer stage, which suggests the clinical value of the strategy [63]. In addition, Lu et al. proposed a modular platform strategy

to sequentially separate PD-L1 positive tumor exosomes with PD-L1 positive no-tumor exosomes based on EpCAM and PD-L1 dual aptamers, achieving discrimination between cancer patients with healthy volunteers [64].

To separate exosomes from complex biological media specifically and nondestructively, Tang et al. proposed a brand-new strategy for exosome isolation based on polyvalent aptamers DNA hydrogel. The CD63 aptamer was placed on one of the DNA chains. After specifically capturing exosomes, DNA chain-2 was added to interact with DNA chain-1 through complementary base-pairing, forming a network that enabled the selective separation of exosomes from breast cancer samples. Moreover, an integrated separation-detection method was developed to integrate molecular beacons and signal probes into the DNA network, allowing the detection of miR-21 and exosome isolation at the same time [65]. This strategy realized the separation of exosomes in complex biological systems and achieved accurate differentiation between clinical breast cancer samples and healthy donor samples. Similarly, Yu et al. designed a substrate nucleic acid probe connected with CD63 aptamer and a DNAzyme probe connected with cholesterol molecule tail respectively, realizing the formation of exosome clusters that could be isolated by normal centrifugation based on complementary connection between the two chains. In addition, the detection system could be cut at specific sites in the presence of magnesium ions, releasing fluorophores to produce a signal for quantitative detection [66].

Paper-based device

Unlike other separation methods, paper-based separation systems usually encapsulate exosome-targeting antibodies on paper-based media, which reduces the cost, providing the possibility for large-scale clinical use (Fig. 1I). Lai et al. deployed anti-CD63 antibodies on filter paper to capture exosomes from colorectal cancer cell lines [67]. Zhang et al. achieved rapid and efficient exosome isolation by using a paper-based device named sEV-IsoPD. A porous membrane was applied to remove the interference of large particles in the sample through the size exclusion method. Then, by covalently coupling CD63 antibodies to a metal-organic framework (MOF) on the paper chip, the sEV-IsoPD system reached a 5.1 times higher yield compared with the ultracentrifugation method. The captured exosomes could be released simply by glutathione hydrolysis for downstream applications [68].

Separation innovation based on nanowire

A nanowire is a nano structure with a diameter of less than 200 nanometers, which is often constructed with different materials and is applied for efficient exosome isolation due to its large surface area (Fig. 1J) [69]. Yokoi et al. innovated the methods for exosome isolation from high-grade serous ovarian carcinoma (HGSOC) patients' serum or ascitic fluids with polyketone-coated nanowires (pNWs). In their previous study, ZnO NWs were used to separate exosomes from urine by electrostatic collection. Al_2O_3 layer was covered on ZnO nanowires to generate a positively charged surface, which showed strong combination ability to urinary exosomes with a negatively charged surface [70]. However, in other body fluids such as serum or ascitic fluids, the dissolution of ZnO NWs caused by external ions often led to failure in exosome isolation. In their recent research, polyketone was coated on the surface of ZnO NWs to reduce their solubility and to avoid adsorbing highly polar compounds. Also, the carbonyl groups of polyketones exhibited better compatibility with exosomes. Compared with ultracentrifugation, the concentrations of exosomes recovered from serum by pNWs reached about 1×10^{11}/mL [71]. Suwatthanarak et al. developed a ZnO nanowire array modified with CD9 peptide to capture exosomes. The exosome could be released under a neutral salt condition for further analysis [72]. In addition, another kind of Au@CuCl2 nanowire was used to connect with CD63 aptamer to capture exosomes from NSCLC serum samples. By adding microporous nanospheres decorated with PD-L1 aptamer into the system subsequently, the device could efficiently realize PD-L1 positive exosomes analysis [73]. Besides, magnetic nanoparticles modified with anti-CD9, anti-CD63, and anti-CD81 antibodies were doped on magnetic nanowires to extract exosomes from plasma within one hour [74].

Separation and enrichment based on lipid

Not only proteins expressed on the exosome surface, lipid on the exosome membrane, such as phospholipid, has been widely used as target molecules for exosome separation and enrichment (Fig. 1K). Pan developed a state-of-the-art plasma exosomal separation platform named EV-FISHER. Based on the metal-organic skeleton and cholesterol modified DNA probe (PSDC), EV-FISHER achieved rapid exosomal capture (10 min) by embedding cholesterol molecules on PSDC into the lipid bilayer of exosomes, which was followed by exosome enrichment via low-speed centrifugation. After that, DNase I was added to hydrolyze the DNA structure on the probe, releasing the captured exosomes, and the separation of exosomes was completed after the supernatant was collected by centrifugation again. The system was proved to detect GPC-1 exosomes from plasma for breast cancer diagnosis with an AUC of 0.835 [75]. Similarly, a lipid nanoprobe was designed to target lipid bilayer structure of membrane for exosome isolation. Based on the combination with microfluidic chips, the device was demonstrated to realize high-throughput enrichment at

over 70% efficiency [76]. Xiang et al. proposed a strategy to combine ultrafiltration with TiO2 nanoparticles to separate exosomes from urine. Utilizing the high-affinity between phospholipid bilayer and TiO2, intact exosomes were isolated from urine within 20 min [77]. However, due to the non-specificity of the lipid bilayer structure, the lipid nanoprobe has difficulty in identifying tumor specific biomarkers.

Several studies constructed separation system by targeting phosphatidylserine (PS) which has been proved to be expressed on tumor-derived exosomes (Fig. 1L) [78]. Xu et al. developed a two-stage microfluidic platform named ExoPCD-chip for exosome isolation and quantitative detection. Samples along with Tim4 modified magnetic beads, which bound to phosphatidylserine on the exosome surface, were added to the chip, achieving rapid exosome capture followed by DNA probe-based in situ electrochemical analysis for CD63 positive exosomes [79]. Similarly, a microfluidic chip with alternating drop-shaped micropillar array was designed to isolate and enrich exosomes by Zheng et al. Using Tim4 bead-based capture method, the device could efficiently isolate tumor-derived exosomes which could be easily eluted by chelating agents subsequently [80]. However, since PS positive exosomes exist in multiple types of tumors, combination of specifically biomarkers is necessary for distinction of different cancer types.

Exosome imaging technology

Compared with classical exosome identification methods, imaging technology has the advantage of showing exosomes in a visual manner which offers great application potential in exosomal tracking, uptake monitoring and special localization. Zong et al. attached CD63 aptamers to silicon quantum dots (Si QDs) to form specifical nanoprobes for exosome recognition. The fluorescence signal was detected by single-molecule localization microscopy (SMLM) for exosome imaging [81]. Puthukodan et al. proposed a method for visual exosome tracking. The CD63-eGFP labelled exosomes were first verified to confirm the labeling efficiency. Then, exosomes were incubated with HeLa cells which were labelled with Alexa647 conjugated anti-CD59 antibodies previously. The uptake process was analyzed by imaging the 3D diffusion trajectories using SMLM [82]. In addition, Liebel et al. integrated digital holography with fluorescent detection system to achieve dynamic imaging of exosomes. By using a fluorescence microscopy, cellular uptake and 3D motion of exosomes could be monitored in a real time manner [83]. McNamara et al. applied direct stochastic optical reconstruction microscopy (dSTORM) to image exosomes labeled with photoswitchable membrane dye. In order to further investigate the distribution of exosomal protein, CD81 and CD9 marked with mCherry and Alexa-488 respectively were imaged by dSTORM, which uncovered the existence of exosomal microdomains [84]. Though the strategies mentioned above exhibited great performance in vitro experiments, whether it possible to directly image exosomes in clinic samples such as plasma and serum remain to be investigated.

Several researches aimed to achieve in-situ exosome imaging in vivo environment. Sancho-Albero et al. realized in vivo exosome imaging by applying magnetic resonance imaging (MRI). MSCs were incubated with PERFECTA emulsion, a molecule with ^{19}F atoms, to produce exosomes which contain PERFECTA and exhibit ^{19}F-NMR signal. After isolating from medium, exosomes were intravenously administered in the mice tail vein. MRI was used for imaging which showed the enrichment of stem-cell-derived exosomes in tumor site [85]. However, the exosomes used in the study were exogenous. In order to construct endogenous exosome imaging models, Verweij et al. injected CD63-pHluorin plasmid which specifically targeted endosomes and expressed on exosomes into zebrafish embryos. Real-time fluorescence imaging revealed the inter-organ communication directed by endogenous exosomes [86].

Artificial intelligence (AI) for cargo analysis

Artificial intelligence (AI) refers to a new technology that utilizes digital computers or machines to simulate and extend human intelligence [87]. Nowadays, AI has been widely used in different areas. In medicine, AI has been utilized to identify infectious diseases that may lead to public health crises and assist clinical patients in disease diagnosis and prognosis evaluation [61, 88].

Machine learning

Wu et al. established an early diagnosis model of three urinary diseases including bladder cancer based on 3D DNA machine and machine learning algorithm. In their research, composite probes were decorated on magnetic nanoparticles to form 3D DNA machine, which could combine with walker strands released from complex at the existence of urinary exosomes. Subsequent digestion induced by exonuclease III and DNA walker, along with rolling circle amplification (RCA) produced fluorescent signals for exosomal protein detection. However, single-biomarker analysis didn't achieve a satisfying sensing performance since the AUC value is between 0.53 and 0.91. With the help of two machine learning algorithm: K-nearest neighbor and support vector machine, multi-biomarkers joint diagnosis realized an average diagnosis accuracy of 95% and 100% respectively, which indicated the great potential of machine learning in disease diagnosis assistance [89]. A method combining DNA points accumulation for imaging in nanoscale topography (DNA-PAINT) with machine learning algorithm analysis

was innovatively proposed by Chen et al. to detect multiple exosomal surface proteins at the single-exosome level. DNA-PAINT is a technology which uses the complementarity of DNA strands to produce fluorescent scintillation [90]. The detection of fluorescence signal subsequently analyzed by machine learning algorithm was realized through total internal reflection fluorescence (TIRF) microscopy. Based on this method, four exosome surface biomarkers including HER2, GPC-1, EpCAM and EGFR were identified to analyze exosomes from healthy controls, breast cancer and pancreatic cancer patients [91]. Diao et al. developed an AuNPs nanomembrane-based SERS system to diagnose breast cancer and cervical cancer with the help of machine learning algorithm. Also, dynamic monitoring of chemotherapeutic process could be realized by profiling exosomal SERS spectra [92]. In addition to proteins, machine learning was also applied in exosomal nucleic acid analysis. A novel exosomal miRNA profiling method was proposed by Lei et al., tumor-specific exosomes were labeled and fused with liposome probes to realize in situ detection of six miRNAs. Machine learning was integrated into the analysis process to achieve accuracy differentiation for metastatic or nonmetastatic prostate cancer with benign prostatic hyperplasia [93]. Nakamura et al. used machine-learning algorithms to analyze exosomal miRNAs to distinguish PDAC patients from healthy controls. Eight miRNAs were selected for diagnosis with a AUC value of 0.98 [94]. A nanosatellite-based miRNA detection strategy was invented by Wen et al., exosomal miRNAs were captured by magnetic beads and analyzed by machine learning algorithm for cancer classification. In clinic samples, the strategy could diagnose five cancer types with 100% accuracy [95].

Deep learning algorithm

As a subset of machine learning, deep learning algorithm integrates neural networks to automatically realize representations learning and analysis with multiple levels of abstraction [96]. Parlatan et al. mixed exosomes from five different cell lines together and tested their components by SERS. After that, deep learning algorithm was applied to identify different exosomes [97]. Shin et al. reported a liquid biopsy method combining AI and SERS, testing the diagnostic performance of the method by using 520 samples including lung, breast, colon, liver, pancreatic and gastric cancer types. Briefly, exosomes from plasma samples were isolated and added to SERS array which contains AuNPs coated on the APTES-functionalized cover glass for detection. After that, deep learning algorithm was used to help analyze SERS spectrum which was mainly assigned to protein constituents to distinguish between different cancer types according to two steps. In the first step, signal was classified as healthy control or cancerous groups to derive a score for the presence of cancer, while in the second step, the consequence was predicted by deep algorithm, which finally identified classification models of plasma exosome signaling patterns in normal and cancer patients [98]. The approach was rapid as the whole step from exosome isolation to SERS detection and analysis could be finished within only 60 min. Additionally, Xie et al. trained deep learning algorithm with SERS spectra from serum exosomes to achieve breast cancer diagnosis and surgical outcome assessment with a 100% accuracy. By combining with data from other cancer types, the system had the potential to be applied for multiple cancer diagnosis [99]. Also, the integration of deep learning algorithm promoted the diagnostic accuracy for lung cancer to 0.93 [100].

For deep learning algorithm, a large sample size is necessary for algorithm optimization to reach more accurate analysis, this may add some difficulty for research. However, once successfully established, the algorithm model can be used not only in one specific cancer type, various other types will also be supported. It is predictable that the application of AI can offer more assistance for the identification of early cancer and the judgment of therapeutic effect, which is helpful to achieve accurate diagnosis and treatment for individuals. Meanwhile, the combined use of AI and other technologies will become a new development trend, bringing new innovations to clinical diagnostic methods.

Technology for detecting cargoes

The detection of cargoes included in exosomes offers great value to cancer diagnosis and treatment monitoring. Here, we summarized both conventional and novel detection methods for different components carried by exosomes (Table 1).

Western blot and enzyme-linked immunosorbent assay (ELISA) are well-known methods for protein detection. However, these two methods have the shortcomings of complex steps and low sensitivity [101]. Colorimetric assay is another regular method to detect exosomal protein [102]. Compared with western blot and ELISA, colorimetric assay such as bicinchoninic acid (BCA) shows the advantages of simpler operation, higher sensitivity and more flexible application. In addition, mass spectrum (MS) has been used for exosomal proteome detection. By using MS, validation for unknown proteins or detection of post-translational modification can be realized [103–105].

In addition to proteins, specific nucleic acids in exosomes may offer diagnostic or prognostic potential in cancer detection [106, 107]. Quantitative real-time PCR (qRT-PCR) is a regular method for nucleic acids detection with high accuracy and efficiency. For high-throughput analysis, next-generation sequencing (NGS) is often

Table 1 Components detected by liquid biopsy

Components	Cancer type	Detection method	Ref.
Proteins:			
HER2	Breast cancer Pancreatic cancer	SPR biosensor aptamer SERS	[91, 123, 125, 133, 190]
CD44	Glioblastoma Breast cancer	LSPR DNA nanoprobes	[130, 190]
CD133	Glioblastoma	LSPR	[130]
EPCAM	Pancreatic cancer Breast cancer	LSPR SERS DNA-PAINT	[91, 129, 133]
EGFR	Pancreatic cancer Breast cancer Glioma	DNA nanoprobes FCM DNA-PAINT	[91, 140, 190]
GPC-1	Pancreatic cancer Breast cancer	DNA-PAINT	[91]
PD-L1	Melanoma Breast cancer NSLLC Liver cancer HNSCC	SPR LSPR SERS FCM	[124, 128, 134, 139, 191]
Nucleic acids:			
PSA mRNA	Prostate cancer	FDT	[174]
PGR mRNA	Breast cancer	ddPCR	[192]
LncPCAT6	Lung cancer	miDER	[158]
LncSLC9A3-AS1	Lung cancer	miDER	[158]
miR-16	Pancreatic cancer	ETFBs	[172]
miR-155	Pancreatic cancer	ETFBs	[172]
miR-1246	Pancreatic cancer	ETFBs	[172]
miR-22	HCC	photothermal dPCR	[157]
miR-15a-5p	Endometrial carcinoma	ddPCR	[159]
miR-451a	Pancreatic cancer	Molecular beacon	[193]
miR-10b	Pancreatic cancer	Molecular beacon ETFBs	[172, 193]
miR-21	HCC, breast cancer, gastric cancer, lung cancer, pancreatic cancer, ovarian cancer	DNA hairpin probes HNCIB ETFBs fLIGHT LDT-CHA	[153, 170–172, 175–177, 194]

conducted. Compared to Sanger sequencing, NGS is able to sequence a mass of nucleic acid at a time [94, 108]. However, these methods have demands for sample concentration, which may cause difficulty in detecting low-expressed nucleic acids. Furthermore, the process of RNA extraction may lead to degradation. In order to overcome these limitations, a large number of new technologies have been developed.

Single exosome detection technology

As conventional bulk detection is hard to reflect the heterogeneity of exosomes, single exosome detection methods have been developed for analyzing both proteins and nucleic acids in recent years, which tends to be a new research direction in the EV area. Wu et al. developed a proximity barcoding assay method to detect surface protein single-exosome level. In their research, oligonucleotides with protein Tags were conjugated with antibodies to form probes which combined with exosomes before their addition to the platform. After being captured in microtiter wells, RCA products were added to hybridize with the probes. Probes bound to the same exosome were connected with the same tag from an adjacent RCA product, which was followed by PCR and sequence reading to obtain protein profiling in single exosome [109]. Ferguson et al. developed a single exosome detection method to detect mutated proteins in pancreatic cancer. By labeling exosomes with fluorochrome-polyethylene glycol-2,3,5,6-tetrafluorophenyl esters (TFP) and fluorescent antibodies, KRAS-mut and P53-mut proteins were detected and analyzed at a single exosome level. Furthermore, a framework mode was established to

connect the amount of circulating tumor exosomes with tumor volume, which predicted that 68% of patients were able to be diagnosable by the method at a tumor size of 0.1cm^3, indicating the potential for early PDAC cancer detection [110]. In addition to protein mutation, the heterogeneity of protein structure, including α-helix and random coil, β-sheet and β-turn, indicates the diversity of cancer malignancy. However, the detection of protein structure tends to be complex and time-consuming, which impedes their clinical application. Xue et al. constructed a near-field infrared (nano-FTIR) spectroscopy system to detect protein structure related to malignancy at a single exosome level. By analyzing infrared spectra of exosomes derived from normal cell lines and tumor cell lines with different levels of malignancy, they found that the occurrence of protein α-helix and random coil in exosomes derived from high malignant tumor cell lines significantly decreased while β-sheet and β-turn significantly increased. In addition, tumor tissue-derived exosomes from breast cancer patients with or without lymph node metastasis further verified the differentiation in protein heterogeneity, which indicated tumor malignancy. The system offered a new perspective to noninvasive cancer diagnosis, which had great potential in clinic application [111]. Ohannesian et al. combined exosome imaging and fluorescence probes to detect single-exosome miRNAs. Exosomes which were incubated with miR-21 hairpin probes were captured on chip decorated with CD9, CD63 and CD81 antibodies. PANORAMA was applied to image exosomes and signals from probes were analyzed for miR-21 profiling [112]. In addition, He et al. constructed a split DNAzyme probe to realize in situ detection of exosomal miR-21 at single exosome level. With the help of Mg^{2+} ions, probes penetrated into exosomes hybridized with miRNA which activated the cleavage of fluorogenic substrate, generating signals for analysis [113].

Several researches developed single exosome detection system that could analyze proteins, nucleic acids or lipids at the same time. Penders et al. presented single particle automated Raman trapping analysis (SPARTA) system to distinguish cancer exosomes from noncancer exosomes with a sensitivity and specificity of more than 95%. By analyzing Raman spectra, differences in exosome cargoes were investigated. A multivariate statistical analysis model was combined with the system, showing significant heterogeneity and higher lipid content in cancer-derived exosomes, which offered evidence for differentiation [114]. Besides, Zhang et al. constructed a ^{siEVP}PRA system based on TIRF to realize simultaneous detection of protein and RNA biomarkers at single-exosome level (Fig. 2). Exosomes were captured on micropattern arrays functionalized with biotinylated antibodies. Then, antibodies and molecular beacons labeled with fluorophore were added to bind to proteins and RNAs. By quantitative detection in situ, the system reached a detection limit which exceeded three orders of magnitude than that of qRT-PCR and ELISA [115].

Antibodies/aptamers combined with electrochemical sensors

Electrochemical sensors measure specific substance by detecting the changes in electrical signal. Electrochemical detections along with antibodies or aptamers were used for analyzing exosomal proteins accurately

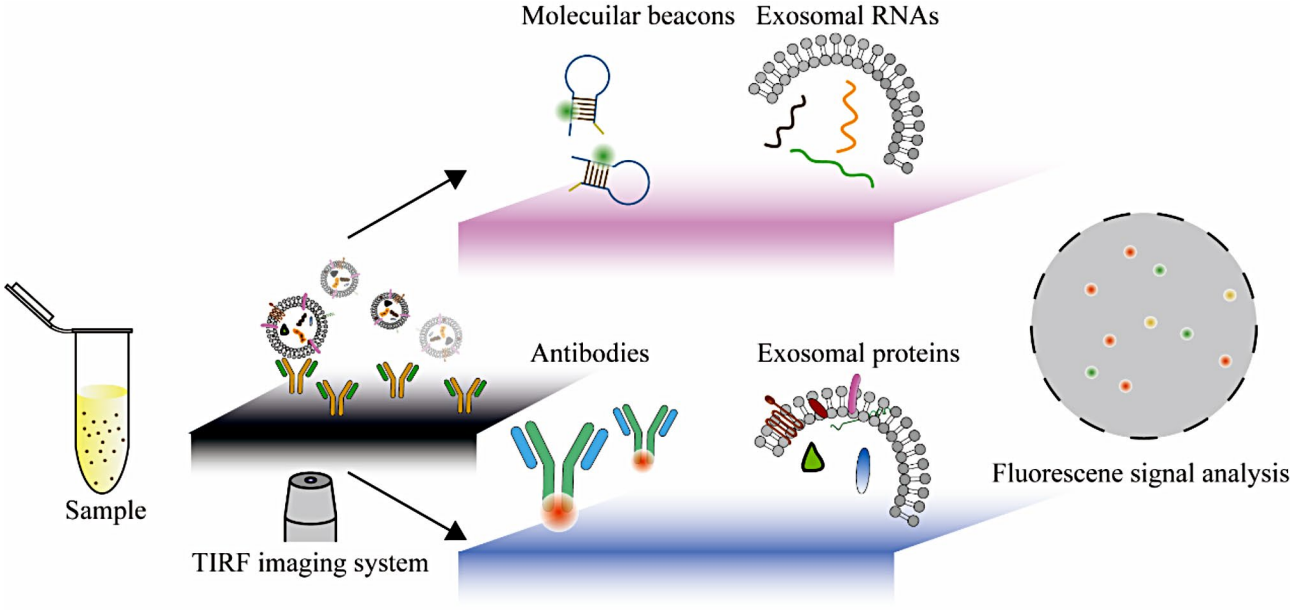


Fig. 2 Single exosome detection technology based on total internal reflection fluorescence (TIRF) imaging system

(Fig. 3A). As glycosylation of proteins often occurs in tumor cells, Jiang et al. developed a dual-recognition technology based on glycosyl imprinting and aptamer to detect exosomal CD63 derived from tumors. In their research, a glassy carbon electrode was decorated with glycosyl-imprinted polymer film to bind to the glycosyl group on exosome surface, CD63 aptamer−bipyridine ruthenium was added subsequently to interact with the glycoprotein. By analyzing electrochemiluminescent signals, the technology reached a detection limit of 641 particles/mL [116]. In addition, Huang et al. introduced a sensitive method to detect gastric cancer exosomes with a detection limit of 9.54×10^2/mL. Briefly, total exosomes were captured by CD63 antibody-modified gold electrode. After that, the MUC3 aptamer, which was connected with primer sequence complementary to the G-quadruplex circular template, specifically bound to gastric cancer exosomes, triggering an RCA reaction. The multiple G-quadruplex units were incubated with hemin solution subsequently to form a hemin/G-quadruplex complex, catalyzing H_2O_2 to produce electrochemical signals for detection [117]. Similarly, You et al. immobilized Ti_2CT_xMXene membranes modified by hierarchical Au nanoarray on electrodes, achieving sensitive detection with a low limit of 58 particles/μL through differential pulse voltammetry (DPV) in the present of EpCAM and CD63 aptamer-bound exosomes [118]. Park et al. developed an integrated device (HiMEX) to realize exosome separation and protein (EGFR, EpCAM, CD24 and GPA33) detection from clinical samples directly. In their study, exosomes were isolated by magnetic beads, exosome-labeling antibodies functionalized with catalysing enzymes were added for electrochemical reactions, realizing protein profiling in 1 h. HiMEX achieved accurate diagnosis for colorectal cancer with 100% of specificity and 96% of accuracy [119].

Plasmonic resonators-based technology

Plasmonic resonators-based technology which mainly contains surface plasmon resonance (SPR) and localized surface plasmon resonance (LSPR) have been widely used in medical fields (Fig. 3B). The SPR phenomenon was first observed by Wood in 1902 [120]. It originates from the resonance between evocation wave and plasma wave on a metal surface [121]. Due to its label-free and real-time feature, SPR has been widely used in biomedical areas [122]. Several strategies have been proposed to improve the sensitivity and detection accuracy of SPR sensor to achieve better performance. Chen et al. constructed a label-free SPR biosensor to detect HER2-positive exosomes with tyramine signal amplification method. Briefly, breast cancer exosomes were captured by molecular aptamer beacon containing HER2 aptamer region, which resulted in the exposure of the G-quadruplex DNA (G4 DNA) followed by peroxidase-like G4-hemin formation. Then, tyramine-coated gold nanoparticles (AuNPs-Ty) on exosome surface deposited in the presence of G4-hemin and H_2O_2, which led to significantly enhanced SPR signal for detection at a range from 1.0×10^4 to 1.0×10^7 particles/mL [123]. Wang et al. proposed a novel method for PD-L1 exosome detection by using Cu-TCPP 2D MOF as a SPR sensitizer, realizing a detection limit of 16.7 particles/mL [124]. Zhai et al. proposed a single tumor exosomal surface protein profiling platform named DISEP to detect exosomes directly from plasma, realizing classification of tumor patients with healthy donners at a ROC of 0.98 [125]. In addition, methods based on plasmonic resonators have been developed to evaluate tumor treatment by detecting drug occupancy and protein composition of exosomes. Pan et al. established an exosome real-time monitoring platform named ExoSCOPE, in which gold nanorings were applied to capture exosomes. Notably, bio-orthogonal probes, which could recruit enzymes to amplify signals by depositing insoluble products were designed to competitively label exosomes. In principle, exosomes with low drug occupancy were labeled with more probes, thus leading to enhancement in plasmonic signals and changes in spectrum. When it comes to clinical analysis, ExoSCOPE realized efficient distinction of responders and non-responders within 24 h after treatment [126].

Different from SPR, using metal film as the substrate, LSPR generally uses nanoparticles or discs located on a glass substrate. Liu et al. decorated silver nanoparticles on gold nano-islands (Ag@AuNIs) and functionalized them with biotinylated CD63, monocarboxylate transporter 4 (MCT4) antibodies for glioblastoma (GBM) exosomes detection. The biosensor realized exosomal detection from serum samples with a LOD of 0.4ng/mL [127]. In addition, a nanoplasmonic sandwich structure was constructed to realize exosomal PD-L1 profiling. Au@Ag nanobipyramids and anti-PD-L1 antibodies were patterned on a glass substrate, which offered a specific binding site to tumor-derived exosomes. Subsequently, anti-PD-L1 antibody-functionalized gold nanorods were added to produce labeling signals, ensuring quantitative detection of PD-$L1^{high}$ exosomes at a LOD of 1.2×10^3 particles/μL [128]. Furthermore, the combination of LSPR with other technologies may further improve the performance. Xiong et al. integrated LSPR with ECL immunosensor to achieve protein detection of pancreatic cancer exosomes at a detection limit of 400 particles/mL [129]. Thakur et al. proposed a new method that combined ultrasensitive TiN–NH-localized LSPR biosensors with atomic force microscopy to detect CD44 and CD133 from glioblastoma-derived exosomes. This work found that TiN-NH-LSPR biosensors could detect and quantify immune-captured exosome levels in the blood and

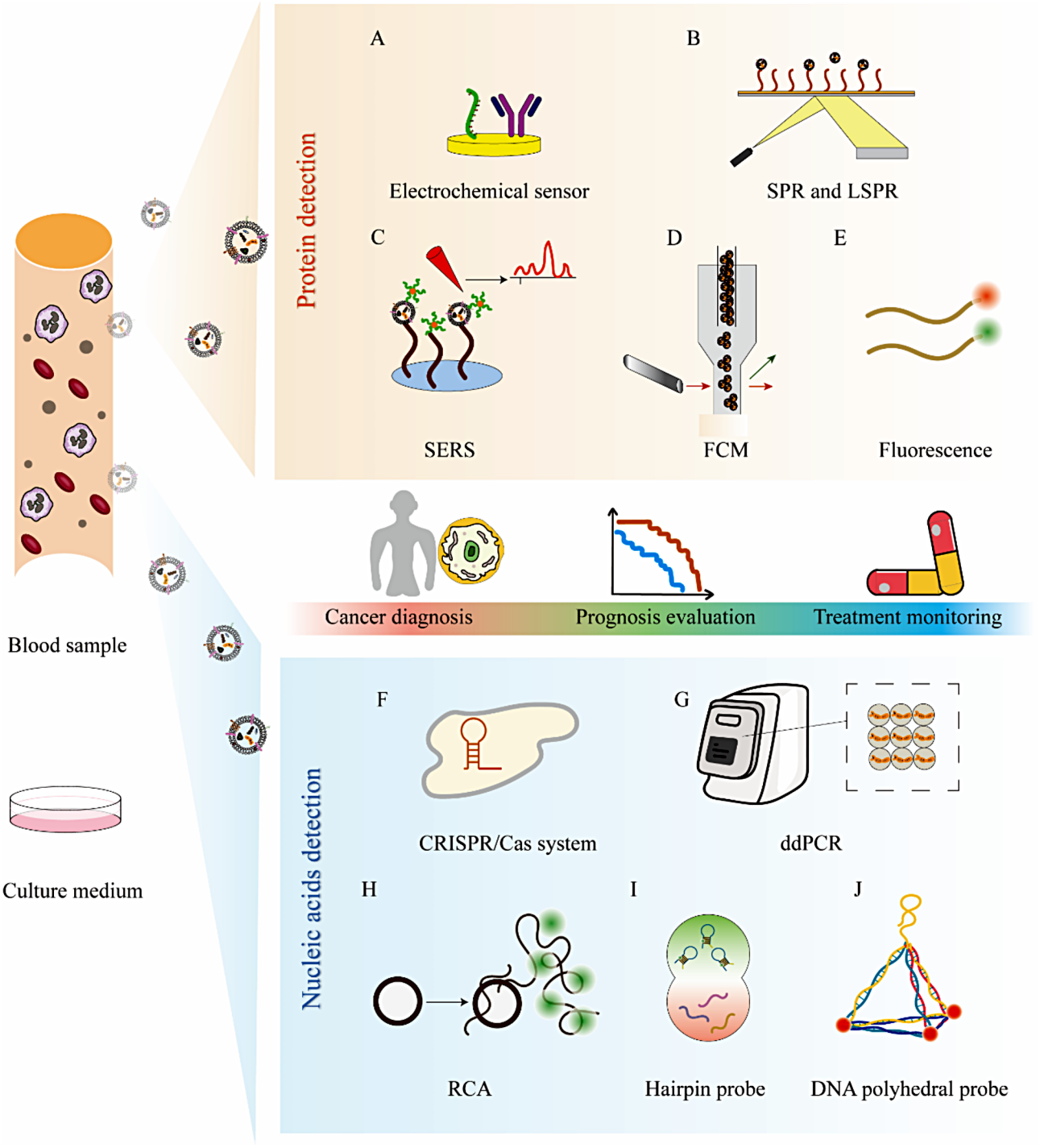


Fig. 3 New technology for analyzing exosomal cargoes. **A**. Using electrochemical sensor to detect proteins. **B**. Using surface plasmon resonance or localized surface plasmon resonance biosensor to detect proteins. **C**. Protein detection based on surface enhanced raman spectra. **D**. Protein detection based on flow cytometry. **E**. Protein detection based on fluorescence signals. **F**. Nucleic acids detection based on CRISPR/Cas system. **G**. Nucleic acids detection based on drop digital PCR. **H**. Nucleic acids detection based on rolling circle amplification. **I**. Nucleic acids detection based on hairpin probes. **J**. Nucleic acids detection based on DNA polyhedral probes

cerebrospinal fluid samples from glioblastoma mouse models at a LOD of 3.46×10^{-3}μg/mL, which supported its further application as a diagnostic method for liquid biopsy [130].

Surface-enhanced raman spectra (SERS)

Surface-enhanced Raman spectroscopy shows significant potential for application in the biomedical field in recent times (Fig. 3C). This is primarily attributed to its notable

advantages, including but not limited to its high sensitivity, uncomplicated sample preparation, rapid detection capabilities, and non-destructive natures. Due to these advantages, SERS has gained significant utilization in the domain of exosome detection. Pan et al. integrated gold nanostars-decorated molybdenum disulfide nanocomposites (MoS2-AuNSs) with nucleic acid aptamers (ROX-Apt) that specifically bind to CD63 proteins present on the surface of exosomes. This novel approach aimed to create a SERS biosensor with enhanced sensitivity and specificity for the detection of exosomes derived from gastric cancer cells. By monitoring SERS signal changes, the sensor enabled a detection limit of 17 particles/μL [131]. Faur et al. employed SERS to discern variations in spectra between salivary exosomes derived from individuals diagnosed with oral and oropharyngeal squamous cell carcinoma and those from the control group [132]. Zhu et al. constructed a SERS aptasensor consisting of hydrophobic assembled nanoacorn (HANA) to detect exosomal proteins. The device was proved to be efficient and highly specific for exosomal HER2, CD63 and EpCAM detection from exosomes in whole-blood samples [133]. In addition, a Fe3O4@TiO2 nanoparticle together with Au@Ag@MBA SERS tag was constructed. Exosomes in serum from NSCLC patients were incubated with Fe3O4@TiO2 nanoparticles, which bound to the exosome phospholipids to realize magnetic separation. Subsequently, Au@Ag@MBA modified with anti-PD-L1 antibody was added for Raman detection. According to SERS signal analysis, exosome PD-L1 levels in healthy donors, patients with early NSCLC and patients with advanced NSCLC could be clearly distinguished, and individual exosome PD-L1 levels could be monitored in real time before and after clinical treatment [134].

A label-free method integrating exosomes separation and detection by the SERS platform was demonstrated by Han et al. Fe3O4/Au nanoparticles, which could be internalized, were incubated with exosomes in supernatant. A magnet was used for separation after 5 hours' incubation, and Raman analysis was conducted subsequently [135]. The advantage of the method lies in its simple and efficient procedure since the use of Fe3O4/Au nanoparticles not only provided a platform for magnetic separation but also enhanced the SERS signals of the exosomes. Su et al. developed a paper-based SERS biosensor to realize diagnosis and molecular subtyping of breast cancer for serum exosomes. Exosomes were injected into the biosensor and incubated with MUC1, HER2 and CEA aptamers added in each test pot on the test pad. SERS probes were added to form sandwich complexes for the protein profiling subsequently [136]. However, since the exosomes used in the research were previously isolated, whether it is possible to incorporate automated isolation device remain to be investigated. Xia et al. replaced the conventional substrate with novel hollow-core anti-resonant optical fibers (HcARFs), which could enhance the Raman signal by more than three orders of magnitude. This device was engineered as a substrate-exosome-SERS probe sandwich structure, and its capability to detect exosomes at the single particle level was demonstrated. Furthermore, a variety of Raman probes were synthesized through the modification of distinct nucleic acid aptamers on SERS probes. This enables the concurrent identification of numerous proteins present on the exosome membrane of both cancerous and non-cancerous cells [137].

Flow cytometry (FCM)

Restricted by the optical diffraction limit, it is difficult for conventional flow cytometry to detect and analyze exosomes [138]. In order to overcome the confines, various improvement measures have been proposed by different researchers (Fig. 3D). Liu et al. constructed a pH-mediated diacyl lipid-conjugated polymers (DLPs) system to convert single exosomes into clusters to meet the standard for protein detection using conventional FCM. Using this pH-mediated assembly system, researchers performed multi-target biomarker analysis for exosomes from multiple cell lines, liver cancer patients and healthy donators, respectively. They found that the combination of MAC-1 with PD-L1 could be used as a new cancer biomarker for early diagnosis of liver cancer [139]. In addition, Wang et al. applied aldehyde latex beads to attach exosomes for FCM detection. Proteins such as EGFR were detected by antibodies bound to exosome surface, which realized the distinction of different malignancy levels of glioma [140]. In a similar manner, Lux et al. isolated exosomes from pancreatic carcinoma cell lines as well as serum samples of PDAC patients. Latex beads were applied to attach exosomes. Analysis of c-Met and PD-L1 were conducted by FCM [141]. In addition, nano-flow cytometer (nFCM) is applied to single exosome detection. Liu et al. used Schirmer test strip to collect exosomes from tears which were isolated by centrifugation and detected by nFCM combined with immunofluorescent labeling subsequently. The expression of CD9, CD63, CD81, CD47, CD45, CD24, and EpCAM was assessed at single exosome level [142]. Morales-Kastresana et al. utilized nanoFACS to detect prostate specific membrane antigen (PSMA) and major histocompatibility complex class II (MHC II) on prostate cancer exosomes and bone marrow-derived dendritic cell exosomes respectively [143]. However, these detection methods rely on expensive instruments, which may limit their clinical application to some extent.

Fluorescence detection

The theory of fluorescence detection is that specific molecules can absorb energy and emit fluorescence. Analysis is conducted based on fluorescence spectrum and fluorescence intensity [144, 145]. Compared with colorimetric detection, fluorescence analysis has the advantages of higher sensitivity and specificity, which promotes their far-ranging use in protein detection (Fig. 3E).

The level of exosomal PD-L1 has been proved to relate to tumor malignancy. However, the detection of PD-L1 based on antibody is often affected by antigen glycosylation. Liu et al. constructed fluorescent anisotropy probes (FSAP) to detect exosomal PD-L1 directly from plasma samples without the need of separation [146]. Furthermore, Huang et al. designed a kind of aptamer that had higher binding efficiency to PD-L1 than an antibody. They combined fluorescence-labeled aptamer with separation-free thermophoresis to realize quantitative detection of exosomal PD-L1 for cancer diagnosis [147].

Chen et al. designed a double-ring trapping probe containing both HER2 aptamer and G4 sequence to realize washing-free detection of breast cancer-derived exosomes. When $HER2^+$ exosomes were present, the aptamer sequences bound specifically to HER2 protein on the surface of exosomes, and the G4 sequence was released at the same time. After hemin was added, G4-hemin could catalyze the substrate tyramine to produce fluorescence, realizing the detection of $HER2^+$ exosomes [148]. The difference in fluorescence signal detected by clinical samples could preliminarily distinguish breast cancer patients from non-breast cancer patients, which has comprehensive clinical value. Liu et al. used fluorescence-labeled aptamer and microfluidic thermophoresis enrichment technology to detect exosome membrane proteins in less than 1μL serum samples. Aptamers firstly identified tumor-associated membrane proteins, and thermophoresis enrichment was then conducted to gather exosomes into center of microfluidic chip, while the free nucleic acid aptamers or proteins remain dispersed. Fluorescence microscope was used to read fluorescence signals to obtain exosome proteomic information which was subsequently analyzed by linear discriminant analysis to realize classification of various cancer types with an AUC of 0.94 [149].

CRISPR/Cas system-based detection

CRISPR/Cas system has been widely known for gene editing [150]. Owing to its sensitivity and precision, the system has been developed for nucleic acid detection (Fig. 3F). Gootenberg et al. proposed the SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) technology in 2017, which marked the birth of CRISPR-based molecular detection technology [151]. Wang et al. constructed a multi-target nucleic acid detection platform combined with RCA and CRISPR/Cas9 system to realize isothermal quantitative analysis of miRNAs in extracellular vesicles. The target was linearly replicated and embedded to form ssDNA amplicons by RCA, which was followed by combination with TaqMan probe to form dsDNA. After the addition of sgRNA/Cas9 complex, dsDNA was cleaved to generate fluorescence. The methodological evaluation showed that the method was in good agreement with RT-qPCR, the gold standard method for miRNA detection, which proves its reliability for application [152]. However, the methods mentioned above were not easy-to-operate since exosomal RNAs have to be extracted before detection. Hong et al. developed an amplification and extraction-free exosomal miRNA detection system by combining CRISPR/Cas13a with liposomes. The CRISPR/Cas13a sensing component was encapsulated in liposomes, which were delivered to exosomes by membrane fusion. By using this method, miR-21-5p in exosomes from ovarian cancer cells and patient plasma was analyzed with a 20-fold higher sensitivity than conventional methods [153].

Dropped digital PCR (ddPCR)

Dropped digital PCR is an absolute quantitative detection method for nucleic acids (Fig. 3G). The reaction system is divided into a large number of independent units for PCR amplification, in which the copy number of nucleic acid is calculated according to Poisson distribution. Several researches applied ddPCR to detect biomarker expression level for cancer diagnosis [154, 155]. Sun et al. purified HCC exosomes to detect 10 HCC-specific mRNA markers quantitatively by ddPCR. The capture and release of exosomes were mediated by covalent chemistry, which contained click chemistry-mediated exosome capture and disulfide cleavage-driven exosome release. RNA of exosomes was extracted, and gene signatures were detected at high sensitivity in distinguishing early-stage HCC from at-risk liver cirrhotic patients with an AUC of 0.87 [156]. In addition, a photothermal dPCR strategy was promoted by Parvin et al. to detect miR-200b, miR-21 and miR-22 in HCC exosomes. Different from the classical dPCR system, the advanced dPCR platform capitalized on SiO2@MoS2@SiO2 nanocomposite to realize PCR thermocycling through photothermal irradiation and fan cooling. The use of gelatin microcarriers exhibited better mechanical properties and avoided the evaporating problems compared with aqueous droplets, enabling a simple and rapid reaction system [157]. Furthermore, a multi-colour fluorescence digital PCR system (miDER) was proposed by Bai et al. to realize efficient lncRNA detection in exosomes from peripheral blood. The expression of SLC9A3-AS1 and PCAT6 was found to show a significant difference between lung cancer and healthy controls, the combined

use of two biomarkers exhibited higher AUC value of 0.811 [158].

The detection of exosomes in plasma has important clinical significance. Zhou et al. validated plasma-derived exosomal miRNA in multiple independent plasma samples using ddPCR. They found miR-15a-5p expressed much higher in endometrial carcinoma patients than in healthy controls, which might serve as a new biomarker for the diagnosis of endometrial cancer [159]. Han et al. used ddPCR to detect plasma exosomal mRNA from PDAC patients. Among 10 candidate mRNAs, SCN7A, SGCD and PPP1R12A mRNAs were finally selected to be the survival prediction biomarker for PDAC patients [160]. Gene mutation correlates with the occurrence of various cancers. Batool et al. constructed a ddPCR platform to detect EGFRvIII mutation in EV-derived RNA with a specificity of 98% [161]. Similarly, Bernard et al. utilized ddPCR to detect KRAS mutations in exoDNA, providing predictive and prognostic information for pancreatic cancer diagnosis [162].

Rolling circle amplification (RCA)

As one of the most commonly used isothermal amplification technologies, RCA has been widely applied for signal amplification both for exosomal protein and nucleic acid detection (Fig. 3H) [117, 152, 163]. Xu et al. isolated breast cancer exosomes which have glycosylated PD-L1 with microfluidic chip. Subsequently, protein recognition aptamer and glycan recognition probes were added to form proximity structure followed by generation of circular DNA structure. Serving as the RCA template, circular DNA mediated the synthesis of DNA amplicon containing hemin/G-quadruplex DNAzyme. Quantification of glycosylated protein has positive relationship with absorption intensity generated by oxidation of ABTS [163]. In addition, Wu et al. used size-coded microbeads to capture tumor exosomes with different biomarkers and the fluorescence signal of which was amplified by RCA with a LOD of 0.0317ng/μL [164]. Gao et al. developed a flow cytometry approach based on RCA with a LOD of 1.3×10^5 exosome/mL. In their work, exosomes captured by magnetic beads were bound to specific recognition aptamers followed by the combined with DNA primers. Fluorescent probe hybridization was conducted based on RCA reaction and using conventional flow cytometry for detection [165]. Similarly, Zhan et al. proposed an RCA strategy triggered by duplex-specific nuclease to detect exosomal miR-21 with a LOD down to 84aM. The amplification products were captured by streptavidin-functionalized terahertz metamaterials to form trimeric complex with AuNPs, which led to a red shift of the resonance peak [166]. Sun et al. detected exosomal miR-92a-3p, a biomarker in colorectal cancer, by periodic RCA. The existence of miR-92a-3p produced the periodic long strands by RCA, which could interact with fluorescence reporters to prevent their adsorption by MOF-525. The fluorescent biosensor realized a LOD of 0.1pM [167]. Different from using metal-organic framework, Yan et al. developed a Cas12-based system to realize sensitive detection of exosomal miRNA. The present of miRNA triggered RCA to form long linear concatemer containing detection zones of Cas12a ribonucleoprotein, activating Cas12a enzyme to cut reporters with fluorophore quencher to release fluorescence signal. The system realized a LOD of 1.35fM, 4.14fM and 7.96fM for miR-196a, miR-451a and miR-1246 respectively [168].

RCA overcomes the cycle process of temperature changes required by PCR reaction, which simplifies the instruments required for experiments and reduces the reaction time. Other technologies can be combined with RCA to develop portable instruments for realizing point of care testing (POCT).

Hairpin probe hybridization

Hairpin probe is a new type of fluorescent-labeled nucleic acid probe with a hairpin structure, which has high sensitivity and specificity (Fig. 3I). In the free state, the fluorophore and the quenching group are close to each other, which quenches the fluorescence. When hybridizing with the target sequence, the spatial configuration changes, and the distance between the fluorescence molecule and the quenching molecule increases, resulting in the recovery of the fluorescence signal [169]. A strategy that fused exosomes with red blood cell membrane vesicles (RVs) containing hairpin probes was proposed by Wu et al. DNA hairpin probes were encapsulated in RVs and delivered by membrane fusion into exosomes secreted by MCF-7 cells to form a restricted space, and the expression of miR-21 was thus detected in situ. This strategy could improve the collision probability between probe and target miRNA and promote the efficiency of DNA self-assembly reaction [170]. Similarly, a nano-bio chip integrated system for liquid biopsy (HNCIB) was proposed by Zhou et al. to realize rapid detection within 6 h. Exosomes were isolated by nano-bio chip, and liposomes containing molecular beacons were added to form exosome-liposome through membrane fusion subsequently. PD-L1 mRNA and miR-21 were detected in exosomes from plasma of lung cancer patients, which showed 1.5-fold differences in comparison with that in healthy controls, indicating the clinical application potential of the system [171]. The methods mentioned above could detect limited number of nucleic acids, which restricted their application. Feng et al. proposed a new strategy taking advantage of encoded-targeted-fusion beads (ETFBs) to detect multiple miRNAs in tumor-derived exosomes at the same time. Plasma samples from pancreatic cancer patients validated the performance of this system.

Through fluorescence-based encoding strategy, miR-21, miR-16, miR-155, miR-1246, miR-10b and miR-196a were detected and used as biomarkers for diagnosis, which showed an accuracy of 98% in comparison with 73% for single miRNA [172].

DNA polyhedral probe

DNA nanostructure, including DNA tetrahedron, DNA cube, DNA icosahedron and so on, is a novel DNA probe self-assembly platform composed of multiple DNA chains, which promotes the efficiency and biostability of probes and exhibits great cellular permeability along with high structural rigidity (Fig. 3J) [173]. Han et al. developed an ultrasensitive assay system using FRET-based DNA tetrahedron (FDT) to realize in situ detection of PSA mRNA from cancer cell-derived exosomes [174]. Similarly, Chen et al. developed fluorescent intracellular-guided hairpin-tetrahedron (fLIGHT) probes to realize the detection of miR-21 in exosomes from non-small cell lung cancer patients at a detection limit of 45.4×10^{-15}M [175]. In order to improve the detection sensitivity, Zhang et al. fixed catalytic hairpin assembly probes (CHA) to DNA tetrahedron, developing a strategy for rapid and sensitive analysis of exosomal miRNAs named localized DNA tetrahedron-assisted catalytic hairpin assembly (LDT-CHA). Four miRNAs, including miR-1246, miR-21, miR-183-5p, and miR-142-5p were detected for gastric cancer early diagnosis with an accuracy of 88.3% and a limit of 25aM, indicating the great potential for clinical applications [176]. Both DNA tetrahedronand DNA cube have been constructed for miRNA detection in exosomes. A double-accelerated DNA cascade amplifier nanostructure (DDCA) consisting of a DNA nanocube and two hairpin DNAs (H1 and H2) modified with Cy3 and Cy5 was promoted by Chen et al. to detect exosomal miR-21. The existence of target miRNA would promote the formation of H1-H2 duplex, accompanied by the release of target miRNA, which would then participate in the new catalytic cycle to cause signal amplification. The system could be used to differentiate between healthy controls and samples of tumor patients at different stages [177]. Mao et al. constructed a novel cubic DNA nanocage-based three-dimensional molecular beacon (ncMB) to detect exosomal miRNAs. The unexpected decrease of fluorescence during the detection of miRNA could be limited due to electrostatic repulsion resulting from the unusual three-dimensional structure [178]. Compared with classical molecular beacons, DNA nanostructure-based probes exhibited higher stability and were easy to be modified.

Clinical translation and application

CTC, ctDNA, and exosomes have been considered as the main contents of liquid biopsy [179, 180]. Compared with CTC and ctDNA, exosomes exhibit higher stability and abundance. Liquid biopsy strategy based on exosomes has raised attention these years both in the research area and clinical application area. Several companies developed commercial products for cancer diagnosis. Exosomics company proposed a peptide-based affinity isolation kit (PA) to isolate plasma exosomes from metastatic melanoma patients, $BRAF^{V600E}$ mutant DNA detection was conducted by digital PCR subsequently. Compared with ctDNA-based liquid biopsy, exosomes isolated by PA exhibited more $BRAF^{V600E}$ mutation, the AUC of PA and ctDNA was 0.72 and 0.66 respectively [181]. CRAIF company developed a commercial system to separate and detect exosomes in urine by using ZnO nanowire device. Within 40 min, 99% of the exosomes could be captured. Machine learning algorithms were applied to analyze exosomal miRNA which realized diagnosis for seven types of cancer in early stage [182]. Biological Dynamics company developed ExoVerita™ Pro exosome enrichment platform to capture exosomes in biofluids based on an ACE chip for downstream analysis. Clinic trial exhibited the performance for stage I and stage II pancreatic cancer diagnosis with an AUC of 0.958 and 0.979 respectively [183]. Nanostics company developed an EV machine learning analysis platform (EVMAP) to diagnose prostate cancer from blood samples. By combining microscale flow cytometry with machine learning algorithm, the platform reached an AUC of 0.75 which demonstrated its potential in cancer diagnosis [184].

EML4-ALK mutant often occurs in patients suffering from NSCLC. In 2016, ExosomeDX company proposed the first exosome-based liquid biopsy products in the world to detect EML4-ALK mutant in exosomal RNA and ctDNA at the same time, which improved diagnostic sensitivity for rare cancer genetic mutations detection, especially in cases where CTC or ctDNA were not easily detectable. Compared with ctDNA analysis alone, the combination detection of exosomal RNA and ctDNA increased the sensitivity by more than three times, from 26–74% [185]. In the same year, ExosomeDX company developed an ExoDx Prostate (IntelliScore) (EPI) test to detect three RNA targets, PCA3, ERG, and SPDEF, in urine exosomes for prostate cancer diagnosis [186, 187]. The AUC for EPI was 0.7, which was higher than 0.56 and 0.62 for PSA and Prostate Cancer Prevention Trial Risk Calculator (PCPT-RC) respectively [188]. In addition, Mursla company synthesized a novel technology platform tool called ExoPheno, which could detect and analyze tissue-specific exosomes in blood samples for cancer diagnosis. With the aim to develop a blood detection method that is faster and more effective than current liver cancer detection standards, Mursla presented a novel exosome-sensitive detection method based on nanoelectronics in 2021 and electrooptic bead nanochip

technology in 2022, both of which have been combined into ExoPheno platform [189]. Although the clinical trial is still underway, the platform has exhibited its great potential in liquid biopsy.

Conclusions and perspectives

In this review, we summarized the most recent technologies developed for exosome isolation, enrichment and cargo analysis methods. Although exosomes-based liquid biopsy has shown its superiority in the early detection, precise diagnosis, prognosis evaluation and therapy monitoring of various cancers, considerable challenges still exist in terms of clinical application. First, since novel exosome separation methods are based on different principles, whether these methods may lead to variation in cargo abundance that will cause bias in content analysis remain to be investigated. Second, the current development of exosome separation and enrichment technology is mostly based on cell culture supernatants from cell lines. Compared with clinical samples such as peripheral blood and urine, culture supernatants have relatively single components and high exosome content. Therefore, considering many devices still face difficulty in achieving a high isolation purity, whether the devices can be well adapted to the complexity of clinical samples remains to be investigated. In addition, the ability of high-throughput operation has to be improved to meet the needs of clinical applications. Third, tumor-derived exosomes are highly heterogeneous. The detection of bulk exosomes may lead to the loss of important information. However, few of the current methods for exosome content analysis can detect at a single exosome level, which may cause poor performance in cancer detection.

Furthermore, the accuracy of detection has to be raised to reduce the occurrence of false negative results. The wide use of AI may offer great convenience to data integration analysis to realize efficient cancer diagnosis. It is foreseeable that the development of new technology for liquid biopsy in the future will be a multidisciplinary process and eventually serve clinical applications.

Abbreviations

CTCs	Circulating tumor cells
ctDNA	Circulating tumor DNA
VAF	Variant allel frequency
CLTA	Clathrin light chain A
HCC	Hepatocellular carcinoma
sEVs	Small extracellular vesicles
NSCLC	Non-small cell lung cancer
PEG	Polyethylene glycol
SAWs	Surface acoustic waves
BAWs	Bulk acoustic waves
DLD	Deterministic lateral displacement
AF4	Asymmetric-flow field-flow fractionation
PDAC	Pancreatic ductal adenocarcinoma
HGSOC	High-grade serous ovarian carcinoma
SMLM	Single-molecule localization microscopy
TIRF	Total internal reflection fluorescence
SPR	Surface plasmon resonance
LSPR	Localized surface plasmon resonance
SERS	Surface-enhanced Raman spectroscopy
FCM	Flow cytometry
ddPCR	Dropped digital PCR
RCA	Rolling circle amplification

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Author contributions

THZ, YD, ZJH and WMY conducted literature collection and writing. THZ, FM, QY drew the figures and tables. ZXX, JRB, GJM and ZX conducted paper revision. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare no competing interests.

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