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Review

Exosomal analysis: Advances in biosensor technology

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ABSTRACT

Exosomes, a subtype of extracellular vesicle secreted by cells, have been a subject of intense research interest. Unfortunately, a simple and reliable method to separate exosomes has yet to be developed. As can be expected, the lack of a standardized method for extraction and purification has contributed to suboptimal inter-laboratory correlation and difficulty in comparison studies. Traditional techniques such as centrifugation, immunoaffinity and size exclusion chromatography, suffer from low purity and tend to be labor intensive thus making their use limited. To mitigate these drawbacks, an integrated biosensor-based exosome separation and detection has recently been developed. In this review, we examine five biosensors that use a variety of detection technology (colorimetric, fluorescent, surface plasmon resonance, surface-enhanced Raman scattering and electrochemical) and propose thoughts on standardization of exosomal analysis.

1. Introduction

Exosomes, released from cells into the extracellular environment, are membrane-bound vesicles containing specific proteins, lipids, and complex RNA, with their normal size at 40–100 nm. They transfer information on the surface of the source cell to the recipient cell, mediating communication between cells [1-3]. Recently, extensive studies on exosomes in cancer have found that exosomes play a key role in tumorigenesis and cancer progression, including immunosuppression, angiogenesis, cell migration, and invasion, and more importantly, they carry information about the tumor microenvironment [4]. Tumor-derived exosomes contain surface proteins and genetic information reflecting their parental cells and can be excreted into body fluids such as serum, plasma, milk, cerebrospinal fluid, semen fluid, and urine with high abundance and stability [5]. Because of these attributes, exosomes are considered applicable to noninvasive early cancer diagnosis and therapeutic efficacy evaluation, and circulating exosomes are becoming a biomarker of "liquid biopsy" for non-invasive cancer diagnosis [6,7]. However, the amount of exosomes secreted by tumor cells in biological fluids is low at the early stage of the disease. Therefore, the ultrasensitiveness and specificity of disease-related exosomes amid their normal cell-derived counterparts have received wide attention, and the rapid development of exosome detection and analysis technology has paved the foundation for exosome research.

Traditional exosome separation and molecular characterization methods have apparent shortcomings, such as more time-consumption, greater loss of exosomes, low separation purity, low yields, low specificity, low repeatability, easy contamination, and high cost. With the development of science and technology, the biosensor-based detection and analysis method has attracted wide attention as one of the latest technologies to improve the sensitivity of exosomes extraction, with its strengths of instant analysis, ease of use, high sensitivity, rapid response, high specificity, and low sample content.

2. Traditional exosome analysis

2.1. Traditional separation method

Traditional exosome separation methods include ultracentrifugation, ultrafiltration centrifugation, polymer sedimentation, immunoaffinity, and size exclusion chromatography. The first method, multistep ultracentrifugation is tedious, consumes a large number of samples, takes more than 10 h, and is inefficient [8]. Besides, exosomes separated this way are often contaminated by other proteins and particles, and

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their structure is seriously damaged. The second method, ultrafiltration centrifugation may cause exosomes loss due to the adhesion of the filtration membrane, and the pressure and shear force during filtration may deform and damage exosomes. As for polymer sedimentation whose common polymer is polyethylene glycol, it may separate non-vesicular pollutants while separating exosomes, and the mixture of the exosome polymer and pollutants would affect downstream analysis. Concerning immunoaffinity, its demand for expensive antibodies, together with the fact that not all cells of the same type express the same target protein for antibodies to bind with, causes its high cost, low reproducibility, and low exosomal production (<50%). Lastly, size exclusion chromatography demands a long time and is unsuitable for large sample processing. Also, the interaction of exosomes with the elution buffer may cause exosomes to degrade or aggregate, resulting in poor fractionation and reduced yield. In a word, traditional exosome isolation methods have their respective shortcomings, affecting downstream detection of exosome-related proteins and nucleic acids (Table 1).

2.2. Molecular characterization

For exosomes isolated, a series of characterization is needed to indicate their physical properties, including their size, morphology, and surface protein content. Common molecular characterization techniques include Scanning Electron Microscope (SEM), Transmission Electron Microscope (TEM), Dynamic Light Scattering (DLS), Nanoparticle Tracking Analysis (NTA), Western Blotting (WB), Enzyme-linked Immunosorbent Assay (ELISA), and Mass Spectrometry (MS). Despite their capability to achieve molecular characterization, these methods still suffer from disadvantages. First, SEM and TEM require a large number of samples to be fixed and stained, so it is difficult to avoid their effects of dehydration and chemical fixatives on exosomes. Meanwhile, DLS measures changes in the intensity of light, but the light intensity signals of big particles would cover those of smaller particles, so DLS is only suitable to measure exosomes prepared by Chromatography with the same size. Then, NTA does not have a recognized standard method [16,17]. As for WB, it requires complex preparation of protein lysate to identify exosomal proteins, and its processing time exceeds 48 h with each measurement consuming more than 2 mL of biological fluid samples; its low separation efficiency and low sensitivity limits clinical research. In terms of ELISA and MS, they cannot detect the very low abundance of exosomes at the early stage of diseases [18]. Given the disadvantages of these characterization methods, new technologies have surged, and their sensitivity and specificity continue to increase (Table 2).

Table 2
Comparison of molecular characterization of exosomes.

Characterization method	Characterization	Advantage	Disadvantage
SEM	Shape	High-resolution	Cumbersome sample preparation steps
TEM	Shape, size	High-resolution	Dehydration and chemical fixatives affect exosomes; unsuitable for rapid measurement of large numbers of exosomes
DLS	Particle size	High sensitivity; simple sample preparation; fast	Not suitable for the measurement of complex exosomes of different sizes
NTA	Size, concentration	No pretreatment required to purify exosomes; high resolution for complex samples	Lack of standardized methods for exosomal analysis
WB	Protein	Provides protein content information	Requires complex protein lysate preparation; long processing time; large sample consumption; low separation efficiency; low sensitivity
ELISA	Protein	Simple; sensitive; specific	Difficult to develop new detection methods and perform simultaneous multiple measurements
MS	Protein	High sensitivity; less sample usage; fast analysis speed; simultaneous separation and identification	Requires large amounts of protein and can't be used for large-scale screening of individual exosomes

3. New methods for exosome isolation and characterization

To reduce the loss and improve the specificity of extracted exosomes, a gentle separation method based on PH response and a new characterization method based on N-glycosyl and viscosity have emerged. PH response-based separation performs precise and gentle exosome separation by the PH response dissociation curve [19]. It reduces damage to exosomes, and meanwhile effectively maintains the integrity of exosome

Table 1
Comparison of traditional exosomes isolation methods.

Separation method	Principle	Advantages	Disadvantages	Purity	Recovery rate
Ultracentrifugation	Settlement coefficient/ Density	Gold standard method [9-11]	Cumbersome steps; large sample consumption; time-consuming; low efficiency; may destroy exosomes	High	Low
Ultrafiltration centrifugation	Ultrafiltration membranes with different relative molecular mass	Fast; easy; no special equipment required [12-14]	Large sample consumption; low yield; exosomes may be lost; exosomes may be deformed and destroyed	High	Low
Polymer sedimentation	Different substances have different solubility in polymers	Fast; easy; small impact on isolated exosomes; PH is neutral	The collected exosomes may be contaminated by polymers to affect downstream analysis	Low	High
Immunoaffinity	Specific capture of antibody-coated microspheres	Point-of-care clinic diagnosis; fast; easy; specific separation; higher separation purity [15]	Antibodies are expensive; high cost; low reproducibility; low yield; only exosomes containing specific protein markers can be collected	High	–
Size exclusion chromatography	Gel pore size and sample molecular size	Not affected by shear forces	Time-consuming; not suitable for large samples; exosomes may be degraded or aggregate; poor separation; low yield	Low	Low

structure and function, thus is conducive to downstream exosomal structure observation and function verification. N-glycosyl and viscosity-based characterization diagnoses related diseases through glycoproteins and glycolipids on the surface of exosomes [20], and observes the particle size of exosomes through membrane viscosity [21]. It avoids dehydration and chemical anfixatives, and helps maintain the exosomal shape, structure, and function. Nevertheless, the drawbacks of both methods could not be ignored. PH response-based separation currently can only be applied to isolate TfR+ exosomes rather than other exosomes, hence the limited clinical application prevents its promotion. As for N-glycosyl-based characterization, despite studies on the N-glycan profile of liver cancer, its data of other diseases is limited. Meanwhile, membrane viscosity-based characterization is employed to analyze the size of exosomes, yet there is to date no recognized data concerning whether differences exist among distinct individuals, tumor cells, or even different stages of the same type of tumor, hence its difficult application. Fortunately, biosensor-based exosome analysis has emerged as the times require, to improve the sensitivity of exosome detection, shorten the detection time, and reduce damage to exosomal structure and function.

4. Biosensor-based analysis of exosome

Recently biosensors have attracted great attention from scientists and technicians worldwide to achieve sensitive detection of exosomes and point-of-care (POC) analysis [22-26]. Biosensors are superior to instrument-dependent technologies due to their affordability, ease of use, rapid response, high sensitivity, excellent specificity, and multiplexing capability [27]. With the development of biosensors, people in resource-limited regions who previously had no access to advanced diagnostic tools can benefit greatly, and cancer therapy can be improved [28]. Hopefully, a significant increase would be witnessed in the use of exosome biosensors in various POC clinical environments.

To develop biosensors, tetraspanins (such as CD9, CD63, CD81, and CD82) or lipid rafts (such as cholesterol, phosphatidylserine, and ceramide) on the exosomal surface are commonly used as the targets for total exosome detection [29]. Meanwhile, cancer-associated antigens on the exosomal surface are used to detect various proteins of different types and determine their cellular origins (such as CEA, EpCAM, HER2, IGFR, LMP1, MUC18, and PSMA). Therefore, these proteins are used as diagnostic and therapeutic indicators for a variety of cancers [30]. Exosomal proteins are effective antigens, and biosensors that are based on corresponding antigen–antibody immune response detect and analyze exosomes through their combination with many methods including colorimetry, fluorescence, surface plasmon resonance, surface-enhanced Raman scattering, and electrochemical methods. Concerning antibodies, aptamers, by virtue of their cost-effectiveness, high stability, and strong resistance, have been used in the past several years as novel biological recognition molecules to replace antibodies, so they are termed “chemical antibodies”. Besides, lipid anchors, such as cholesterol and oleyl ether anchors, are a new bio-recognition method insertable into the lipid bilayers of exosome membranes through hydrophobic interactions. It has excellent efficiency and is not affected by the number of proteins on the exosomal surface, hence its application for biosensors development [31-33].

4.1. Colorimetric biosensors

4.1.1. Paper-based colorimetric biosensors

Paper-based colorimetric biosensors generally use paper as the substrate. Recently, lateral flow biosensors (LFBs) have been reported which generally have four components: a sample pad, a conjugate pad, a nitrocellulose membrane containing test and control lines, and an absorbent pad [34]. The sample solution migrates by capillary action and is passed over all of these pads. The targeting exosomes are captured by the capture elements fixed on the test line entailing antibodies, and then the antibody detection elements labeled by signal elements such as nanoparticles and enzymes are attached on the exosomes to form sandwich complexes, resulting in visible color changes due to accumulation of nanoparticles on the test line.

4.1.2. Solution-based colorimetric biosensors

Solution-based biosensors use gold nanoparticles (AuNPs) that display red color as the colorimetric signal. Jiang [35] and colleagues reported a biosensor platform using aptamers to complex with AuNPs in a high-salt solution so that AuNPs do not aggregate. In the presence of exosomes, the strong specific binding between the exosomal protein and the corresponding aptamer forces the latter to leave the surface of AuNPs, leading to the aggregation of AuNPs, thus the color of the solution changes from red to blue and such a change can be detected by ultraviolet absorption spectroscopy.

He et al. [32] used a cholesterol-based DNA chain hybridization (HCR) to realize sensitive detection of exosomes from HepG2 cells using immunomagnetic beads with an anti-CD9 antibody. Then cholesterol-based DNA probes were introduced. Since exosomes have a lipid bilayer structure, cholesterol can be inserted into the bilayers, thereby connecting the DNA probe to exosomes to initiate the HCR reaction. Next, horseradish peroxidase (HRP) was introduced into the reaction system via a streptavidin–biotin interaction. HRP can catalyze the reaction between H_2O_2 and 3,3′,5,5′-tetramethylbenzidine (TMB) so that TMB will be oxidized, and the solution color will change from colorless to blue. Finally, semi-quantitative detection of exosomes can be achieved by detecting the change in absorbance of the solution through ultraviolet absorption spectroscopy.

Nonetheless, using a natural enzyme as the signal tag has many disadvantages, including its demand for a complicated purification process and cautious storage conditions. Recently, various nanozymes that mimic activities of natural enzymes have gained widespread attention from biosensor manufacturing, due to their advantages including low cost, bulk preparation capability, and high resistance to pH and temperature changes. Xia et al. [36] used the interaction between single-walled carbon nanotubes (s-SWCNTs) and aptamers to detect exosomes. The aptamers recognizing CD63 were mixed with s-SWCNTs and absorbed on the surface of s-SWCNTs to improve the catalytic performance of s-SWCNTs to effectively catalyze the H_2O_2-mediated oxidation of TMB, resulting in the change in solution color from colorless to blue. If exosomes are present, the aptamers will specifically bind to the CD63 protein on the exosomes by leaving the surface of s-SWCNTs, thus reducing the catalytic performance of s-SWCNTs. With an increasing concentration of the CD63-positive exosomes, the solution changed significantly from dark blue to light blue, and the exosomal content could be semi-quantitatively analyzed by ultraviolet absorption spectroscopy.

Colorimetric biosensors are of great importance for practical POC applications because of their fast speed, easy operation, small sample size, and low cost. Typically, colorimetric biosensors can help observe changes in color with naked eyes easily and instantly, and they generate a ‘yes/no’ answer or a semi-quantitative result without any additional analytical instrumentation. Moreover, this method can be used with different aptamers to achieve a specific semi-quantitative analysis of the content of multiple proteins on the exosomal surface. In this way, separation and detection of exosomes are integrated, and such a simplified process is easy to operate.

4.2. Fluorescent biosensors

When substances emitting fluorescence are modified on an exosome surface, the intensity of the fluorescence can be used for qualitative or quantitative analysis. Fluorescent biosensors show great advantages thanks to their ability to amplify signals and their high detection sensitivity. There are three types of fluorescent biosensors: paper-based, solution-based, and micro-platform-based.

4.2.1. Paper-based fluorescent biosensors

Chen and colleagues reported their development of a fluorescent paper-based analytical device (PAD) using UCNPs and AuNRs as a fluorescence-quenching nanopair to detect exosomes from hepatocellular carcinoma cells (HepG2) [37]. The CD63 aptamer sequence was split into two different fragments: capture probe (CP) and detection probe (DP), to capture exosomes and form a sandwich structure. UCNPs and CP were preloaded onto the sodium periodate-oxidized filter paper to generate green luminescence. AuNRs modified by DP were mixed with exosomes and aliquoted onto the filter paper so that the formed sandwich structure reduced the distance between AuNRs and UCNPs, thereby quenching the green luminescence through luminescence resonance energy transfer. The concentration of exosomes was quantified by the luminescence intensities: the higher the exosome concentration, the weaker the green luminous intensity.

4.2.2. Solution-based fluorescent biosensors

Solution-based fluorescent biosensors are typically based on the formation or release of fluorescent nanoparticles or fluorophores. He [33] proposed a three-step method based on fluorescent copper nanoparticles (CuNPs) to detect exosomes simply and quickly: First, exosomes are captured by cholesterol-modified magnetic beads (MBs). Then these exosomes were specifically recognized by the aptamer-modified copper oxide nanoparticles (CuO NPs) to form the magnetic bead-exosome-copper oxide sandwich structure. Finally, this sandwich complex from the previous step was dissolved by nitric acid, and copper oxide was converted to Cu^{2+} which in turn was reduced into Cu fluorescent nanoclusters (CuNPs) by sodium ascorbate in the presence of polythymine. With the increase of reduced CuNPs, the fluorescence intensity also increased and was proportional to the exosome concentration, thereby realizing the detection of exosomes.

Another solution-based fluorescent biosensor is based on the release of fluorophores [38], whereby fluorophore-labeled aptamers are used and their fluorescence is quenched by graphene oxide (GO). However, these aptamers would be released from GO when in contact with exosomes, so that their fluorescence signal is enhanced.

4.2.3. Microplatform-based fluorescent biosensors

Tian and colleagues presented a digital exosome detection microchip integrated with nucleic acid-based amplification [31]. Its principle is to synthesize a phospholipid-like molecule BAM-DNA molecule from the BAM insertable into the phospholipid molecule layer and a synthetic DNA molecule, and then to label exosomes using BAM-DNA. Since the labeled and purified exosomes carry the target DNA molecules, they could be detected via the detection of the DNA molecules by nucleic acid. One of the innovations of this method lies in its output method. The labeled exosomes are assigned to chambers in the chip. After the nucleic acid amplification reaction, chambers with exosomes would present a positive signal and are indicated by the number "1″, while those without exosomes presenting a negative signal are recorded with the number "0". Quantitative analysis of exosomes can be achieved at the single-particle level by counting these numbers.

Nevertheless, the performance of current microfluidic methods is restricted by fundamental limits including boundary conditions, microscale mass transfer, and interfacial exosome binding. Zhang et al. reported a comprehensive strategy of multiscale integration by designed self-assembly (MINDS), addressing these three limits simultaneously in one device [39]. MINDS was used to combine micro-patterning with a 3D nanostructured herringbone mixer, and then an anti-CD81 monoclonal antibody was employed to capture overall exosomes. Meanwhile, specific monoclonal antibodies were used to detect surface protein markers. Finally, detection sensitivity was enhanced by fluorogenic signal amplification.

It is worth mentioning that nanostructured herringbone (nano-HB) could be made of various nanomaterials, and that complex morphology can be created by controlling assembly variables (including the particle size ratio and volume fraction) [40,41]. Experiments have shown that 3D nano-HB can promote microscale mass transfer by disrupting the laminar flow in microchannels [42,43], increase binding efficiency and speed by increasing surface area and probe density, and permit drainage of the boundary layer of fluid through the pores of a nano-HB, thus reducing near-surface hydrodynamic resistance [44,45] and promoting particle–surface interactions for exosome binding.

All in all, fluorescent biosensors use luminescent groups to amplify the signal to detect exosomes, and then semi-quantitatively analyze exosome amounts through changes in fluorescence color. In this way, ultra-sensitive detection of tumor-associated exosomes is realized to assist the diagnosis of early tumors.

4.3. Surface plasmon resonance biosensors

Surface plasmon resonance (SPR) is an optical phenomenon used to track interactions between biological molecules in real time. Its detection principle is to first bound one kind of biomolecule (target molecule, such as an antibody) to the gold surface of the biosensor, and then inject a solution of another biomolecule (analytes, such as exosome) that can interact with the target molecule into the biosensor, so that the solution flows through the gold surface. The combination of biomolecules increases the mass of the biosensor gold surface, leading to a proportional increase in the refractive index of the gold surface, resulting in monitorable changes in the reaction between biomolecules. Besides, given the comparability between the surface plasmon wave depth (<200 nm) and the size of exosomes, SPR-based biosensors are suitable for exosome research.

Reported substrates of SPR biosensors are mainly based on gold chips. To enhance the SPR signal or explore more detection information, a two-step strategy is used in some studies injecting additional components onto the gold chip. Im and colleagues first pre-functionalized the gold surface of 12 microfluidic channels with polyethylene glycol and corresponding antibodies [7]. After exosomes were captured by antibodies, the secondary labeling of the biosensor was done with gold nanostars, resulting in optimization of SPR signal by 300%. Picciolini and colleagues also designed a two-step SPR biosensor to detect multiple exosome subpopulations of neural origin directly in human plasma [46]. They first used a series of antibodies to isolate and detect exosomes from neurons and oligodendrocytes. Then, the signal was enhanced and the components of CD81 and GM1 of exosomes of each subgroup were quantified by injecting secondary antibodies (anti-CD81 and anti-ganglioside GM1) on the fixed vesicles. Their study demonstrates that exosomal proteins are not expressed uniformly, but show variable abundance depending on the exosome cellular origin.

In addition to conventional gold chips, Thakur and colleagues [47] also introduced self-assembly gold nanoislands (SAM-AuNIs) chips without any antibody functionalization to detect and distinguish exosomes in MVs isolated from SH-SY5Y cells, A-549 cells, and mouse blood serum. They found that SAM-AuNIs chip without any antibody functionalization could generate a localized SPR response to exosomes instead of other MVs, indicating a higher specific biophysical interaction between the exosome membrane and the chip. Therefore, the biophysical detection of exosomes can be performed directly with the naked SPR biosensor without modification of antibodies.

To summarize, SPR technology has great advantages for its real-time detection, no requirement for sample labeling, low sample requirements, high sensitivity, high throughput, and no background interference.

4.4. Surface-enhanced Raman scattering biosensors

Surface-enhanced Raman scattering (SERS) biosensors can be divided into two types: solid-based and solution-based.

4.4.1. Solid-based SERS biosensors

Solid-based SERS biosensors have two main parts: a solid substrate and a SERS nanoprobe, both with a recognition element, and these two parts can form sandwich structures with exosomes. In recent studies, the sensitivity of SERS biosensor substrates has been improved by being coated with a rough nanoscale metal layer. Also critical to the performance of biosensors is the SERS nanoprobe with three components: a metal core with various shapes and sizes, a Raman reporter molecule, and a recognition element. By self-polymerization reaction, Li and colleagues developed a SERS nanoprobe consisting of a gold metallic core coated with silver, a Raman reporter molecule pATP, and an antibody as the recognition element [48]. Their substrate was modified by PDA to spare enough space to capture antibodies, and the detection limit of one exosome in 2 μL of sample solution was about 9×10^{-19} mol/L, thus realizing ultra-sensitive and specific exosome detection. Kwizera et al. changed the shape of the gold metallic core and created a SERS nanoprobe based on such nanoparticles [49]. In their design, AuNRs coated with the organic dye QSY21 were loaded on the glass substrate coated with gold. The negatively charged exosomes (zeta potential being about −10 mV) and positively charged AuNRs (zeta potential being about + 35 mV) were bound through electrostatic adsorption. The efficiency and sensitivity of such non-specific binding can be enhanced without the need for specific protein.

4.4.2. Solution-based SERS biosensors

Solution-based SERS biosensors often use magnetic nanoparticles as substrates because they can separate the exosome-linked sandwich structure from complex samples by applying an external magnetic field. Besides, the magnetic nanoparticle-substrate has a larger surface area than the glass slide-substrate, thus it can capture exosomes directly in the solution with higher efficiency and sensitivity. Zong et al. synthesized a magnetic nanobead substrate by coating Fe_3O_4 nanoparticles with a silica shell and antibodies. Then nanoparticles were used as the base and Au@Ag nanorods@DTNB@SiO_2@antibodies as the SERS nanoprobe to form a sandwich complex to detect exosomes [50]. Replacing antibodies with aptamers, Wang et al. prepared magnetic

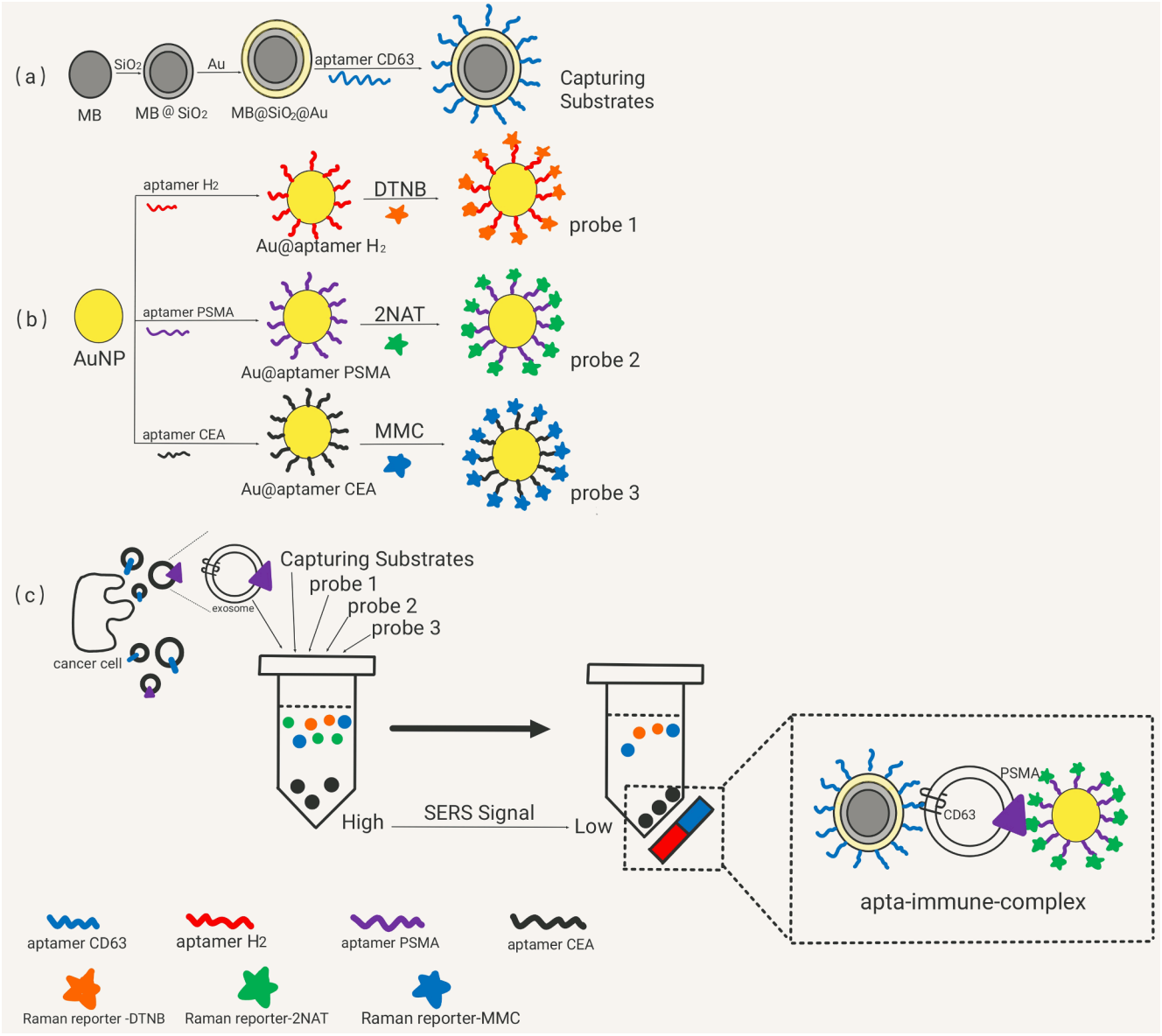

Fig. 1. Preparation of exosome capture substrate: magnetic beads (MB) are coated with the first layer of SiO2 shell (MB@SiO2) and the second layer of gold (MB@SiO2@Au), and are modified with an aptamer of the surface protein CD63 to prepare an exosome capture substrate (magnetic nanobeads@SiO2@Au@CD63-aptamer).

nanobeads@SiO_2@Au@CD63-aptamer as the capturing substrate, and matched three different aptamers with the Roman reporter gene to cover the gold nanoparticles and assemble three kinds of SERS probes, realizing the simultaneous detection of exosomes secreted by breast cancer cells SKBR3, prostate cancer cells LNCaP and colon cancer cells T84 [51]. (Fig. 1)

(a) Preparation of SERS probes: different aptamers are matched with different Raman reporter genes to be coated on the gold nanoparticles to form three different AuNP@aptamer@Raman reporter gene SERS nanoprobes. SERS probe 1 whose Raman reporter gene and aptamer are DTNB and aptamerH2 identifies exosomes derived from breast cancer cells SKBR3. SERS probe 2 whose Raman reporter gene and aptamer are 2NAT and aptamerPSMA identifies exosomes derived from prostate cancer cells LNCaP. SERS probe 3 whose Raman reporter gene and aptamer are MMC and aptamerCEA identifies exosomes derived from colon cancer cells T84.
(b) Separation and detection of exosomes using SERS biosensor: magnetic nanobeads@SiO_2@Au@CD63-aptamer are used as the capture substrate to identify the exosomal CD63 surface protein to capture exosomes secreted by tumor cells. Three different AuNP@aptamer@Raman reporter gene SERS nanoprobes are added to the target exosomes to form a sandwich structure of the capture substrate-exosomes-SERS probe. When an external magnetic field is applied, the sandwich structure connected to the exosomes is magnetically precipitated, and the detection of a decrease of SERS signal intensity in the supernatant indicates the presence of target exosomes. SERS probes 1, 2 and 3 realize the simultaneous detection of breast cancer cells SKBR3, prostate cancer cells LNCaP, and colon cancer cells T84 derived exosomes, thereby, simulating cancer screening.

Pang et al. developed a strategy combining Fe_3O_4@TiO_2 with SERS tags for high-speed separation of circulating exosome and precise detection of the exosomal PD-L1 biomarker [52]. In their initial step to isolate exosomes, Fe_3O_4@TiO_2 nanoparticles were designed and then added to the serum filtered through a syringe-driven filter to be incubated with the serum. At this stage, exosomes can be arbitrarily captured through the binding of TiO_2 and hydrophilic phosphate of the exosomal phospholipids [53], and then be isolated from the serum by a magnet within 20 s. In the second step to detect the exosomal PD-L1 biomarker, an "Au@Ag@MBA" SERS tag modified by an anti-PD-L1 antibody was added for exosomal PD-L1 labeling and SERS detection. The PD-L1 level can be quantified by the SERS intensity of the characteristic peak at 1074 cm^{-1} of the SERS tags. It can be seen that the SERS intensity at 1074 cm^{-1} was enhanced with the increased exosome concentrations, and that there was a good linear relationship between the SERS intensity and the logarithm of the exosome concentration. Using this strategy where no antibody or aptamer is needed, the content of Fe_3O_4@TiO_2 is 0.8 mg within the incubation time of 5 min, and the exosome capture efficiency is as high as 96.5%. Moreover, this strategy requires just 4 μL of clinical serum samples to accurately quantify exosomes PD-L1 within 40 min.

In summary, the surface-enhanced Raman method has its advantages to achieve rapid exosome analysis and detection thanks to its repeatability, stability, simple operation, small sample size, high spectral resolution, sensitivity to single-molecule levels, and the ability to detect multiple exosomes simultaneously.

4.5. Electrochemical biosensors

Electrochemical (EC) biosensors can convert the recognition of biomolecules into electrical signals, including current, potential, and impedance. With the development of integrated circuit technology and the emergence of new electrodes, EC biosensors are developing in the direction of miniaturization and portability. Specifically, there are three types of EC biosensors: conventional, screen-printed, and micropatterned electrode-based.

4.5.1. Conventional electrode-based EC biosensors

Conventional electrode-based EC biosensors primarily use glassy carbon electrodes or gold electrodes. Boriachek and colleagues reported electrochemical stripping voltammetry, whereby bare glassy carbon electrodes were used as substrates, and dissolution of metals from CdSe quantum dots (CdSeQDs) was employed as signals to detect exosomes [54]. First, MBs modified by tetraspanin antibodies (primary antibody, such as CD9 or CD63) were used to capture exosomes. Subsequently, biotin-modified CdSeQDs were combined with HER-2 and FAM-13B antibodies (secondary antibodies) as markers for breast and colon cancer. These secondary antibodies form sandwich structures with previous exosomes and primary antibodies. After washing and purification, retained CdSeQDs were dissolved in HNO_3, and then the content of Cd^{2+} was detected by anodic stripping voltammetry on bare glassy carbon electrodes. By detecting the signal of Cd^{2+}, quantitative detection of different tumor exosomes can be achieved, and this method enabled the highly sensitive detection of 100 exosomes/μl.

Besides glassy carbon electrodes, gold electrodes are also commonly used as candidate substrates because of their high sensitivity, rapid response rate, and fast mass transfer rate. Huang et al. reported a label-free aptamer sensor for the selective and sensitive detection of gastric cancer exosomes by combining the hemin/G-quadruplex system and rolling circle amplification (RCA) [55]. First, different kinds of exosomes were captured in the gold electrodes modified with anti-CD63 antibodies. Among these exosomes, only specific aptamers of gastric cancer exosomes can be linked to primer sequences complementary to the G-quadruplex circular template, thus triggering RCA and generating a large number of G-quadruplex units. These G-quadruplex units were then incubated with hemin to form hemin-G-quadruplex structures, which catalyzed the reduction of H_2O_2 and meanwhile produced electrochemical signals. This aptamer sensor showed high selectivity and sensitivity to gastric cancer exosomes with its detection limit at 9.54×10^2 mL^{-1}. Also, since RCA reaction can be performed at room temperature, and integrity of exosomes could be ensured, this electrochemical aptasensor is expected to become a useful tool for the early diagnosis of gastric cancer. (Fig. 2)

4.5.2. Screen-printed electrode-based EC biosensors

Screen-printed electrode-based EC biosensors have recently attracted wide attention because of their low cost and flexibility compared with conventional electrode-based EC biosensors. Jeong and colleagues combined magnetic enrichment, antibody recognition, and enzymatic signal amplification with a screen-printed electrode for high-throughput measurements [56]. First, the surface of the magnetic beads was modified with tetraspanin antibodies to selectively capture exosomes in the body including serum, plasma, and urine. Then, secondary antibodies labeled with HRP were added to the signal unit and specifically bound to proteins on the exosomes. Finally, the oxidation of TMB was catalyzed by an enzyme to amplify the electrochemical signal. Wang and colleagues developed a DNA nanotetrahedron (NTH) sensor to directly capture and detect exosomes from liver cancer cells [57]. The electrodes were modified with the tetrahedron DNA whose structure contained the DNA sequence of the aptamer, so the spatial orientation could be maintained, the obstruction effect could be reduced, and exosomes be identified more specifically. Compared with traditional aptamer-based biosensors, the sensitivity of this method is increased by 100 times.

4.5.3. Micropatterned electrode-based EC biosensors

Due to the miniaturization of micropatterned electrode-based EC biosensors, exosomes can be detected using a small sample volume. Zhou and colleagues immobilized aptamers on the surface of micropatterned electrodes, with one end of the aptamers hybridized with the

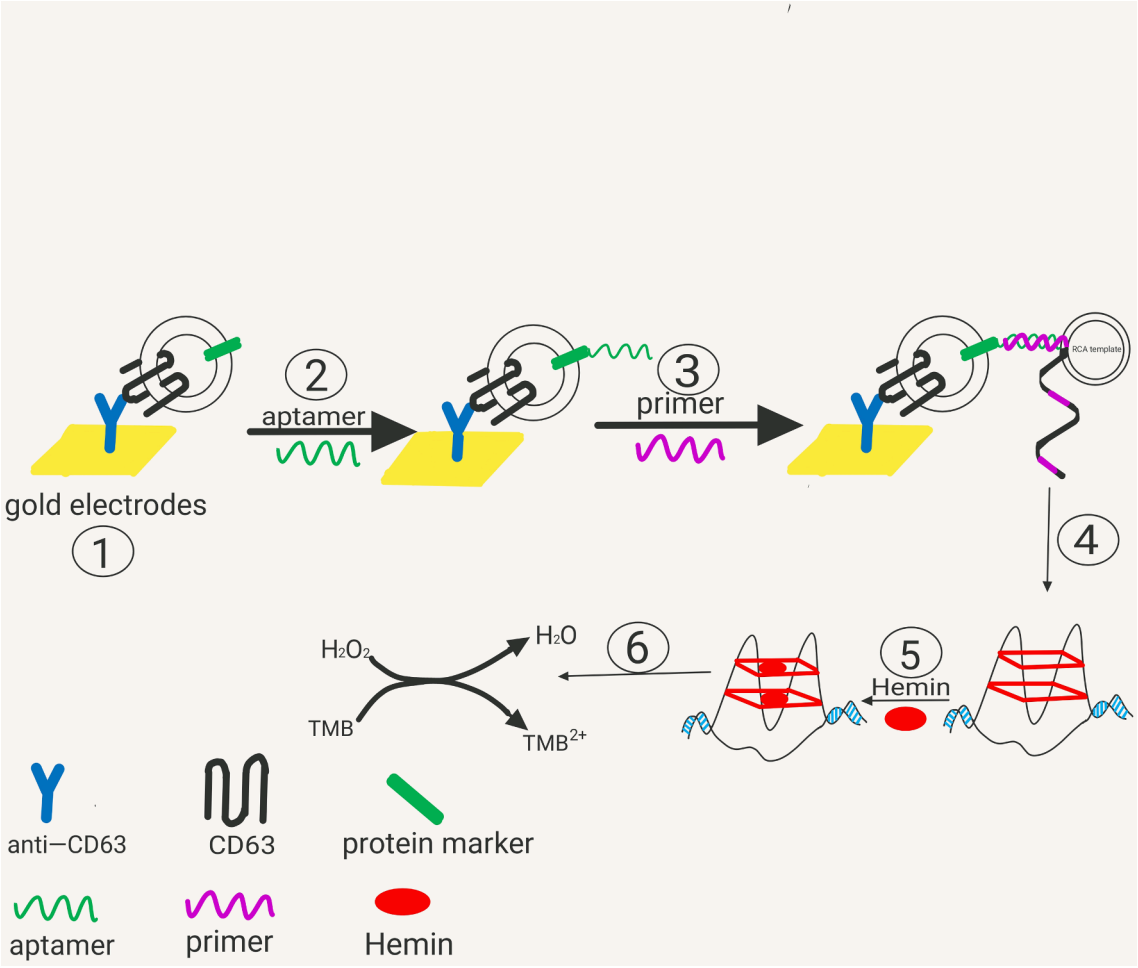

Fig. 2. A gold electrode modified with the anti-CD63 antibody is used to capture exosomes. ②Specific aptamers of gastric cancer exosomes are selected to bind to the transmembrane proteins of exosomes to make the probes. ③ The specific aptamers of gastric cancer exosomes are connected to the primer sequences that are complementary to the G-quadruplex circular template. ④ Rolling circle amplification is triggered at room temperature to produce a large number of G-quadruplex units. ⑤ The product from the previous step is incubated with hemin to form the hemin/G-quadruplex structure. ⑥ A series of H_2O_2-involving reactions is catalyzed by the hemin-G-quadruplex structure to produce electrochemical signals.

redox-modified DNA strand. When the target exosomes existed, the CD63 aptamers could recognize the CD63 protein on the exosomal membrane, release the probing strands with redox reporters, and reduce EC signal, thus realizing exosomal detection [58].

In short, clinical applications of traditional exosome separation methods have been limited due to their lack of sensitivity, separation efficiency, and purity. Also, new methods like PH-based separation are difficult to promote. So far, high-sensitivity detection of exosomes has been achieved with the help of biosensors to specifically recognize exosomal surface proteins using antibodies or aptamers and multiple detection methods. However, these biosensors also have their respective advantages and disadvantages. (Table 3)

5. Conclusion and outlook

Either exosome isolation or its detection is still in the experimental stage and has not yet been clinically applied. The key obstacle to transforming exosome research results into clinical practice is the lack of standardized technical specifications, including the selection of specimens before testing, a uniform standard while testing, and every link after testing. The International Society for Extracellular Vesicles (ISEV) is working towards improvements for the standardization and reproducibility of extracellular vesicle (EV) research to improve the comparability of results among institutions. They proposed a roadmap for collecting, handling, and storing EVs from blood [59]. The first step is to collect, handle and store biological samples, then to carry out sample quality control procedures, and finally to use samples of known quality for downstream applications and public reports.

Pre-analytical quality control is the most important part, although it is done before the test specimen enters the laboratory. As blood is the most commonly used biological fluid for EVs research, it is indispensable for pre-analysis quality control to improve the rigor and reproducibility of blood collection and handling. Nevertheless, even a detailed standard operating procedure (such as plasma preparation) does not guarantee that the prepared samples are comparable among individuals and laboratories, therefore, opinions on the quantitative description of plasma or serum are needed. Aled Clayton et al. [59] proposed that if the goal of blood centrifugation protocols is to prepare platelet-depleted plasma, then why not measure and quantify the residual platelet count in the "platelet-depleted" plasma to monitor the efficacy of the centrifugation procedure? Quantitative descriptions of prepared samples shed insights into the quality and consistency of the prepared plasma or serum samples, and establish a reliable biorepository for future research on EVs. Measuring sample quality before the analysis of EVs will directly help understand the efficacy of previous blood collection and handling protocols. Moreover, specimen quality monitoring and transparent reporting will improve the rigor and ultimate reproducibility of downstream analyses.

Table 3
Comparison of biosensors for exosome detection.

Types	Benefits	Limitations	Perspectives
Colorimetric Biosensors	Fast response; easy operation; no need for large samples; cost-effective; POC application	Low sensitivity	(1) Portable, miniaturized and economical (2) High intelligent and integrated
Fluorescent Biosensors	High sensitivity	Large instruments; expensive; large background signals; fluorescent labeling may weaken binding	(3) High sensitivity, high accuracy, and high stability (4) Development of nanoparticles and nanoenzymes
Surface Plasmon Resonance Biosensors	Real-time detection; label-free sample; no need for large samples; high sensitivity; high throughput; no background interference	Testing cost, stability, and testing efficiency need to be improved	
Surface-Enhanced Raman Scattering Biosensors	Repeatability; stability; easy operation; no need for large samples; high spectral resolution; sensitive to single-molecule level; can detect multiple exosomes at the same time; fast response; cost-effective	Expensive equipment; manufacturing of SERS probes	
Electrochemical Biosensors	Simple and miniaturized equipment; cost-effective; fast response; high sensitivity; wide measuring range	Poor repeatability	

Any clinical test needs to be based on the validity of the test methods, and the ideal situation would be to have standardized samples for comparison. However, there are barely standardized exosome samples, and nearly all exosomal diagnostic research is based on self-built detection methods and detection intervals. Besides, detection with high-sensitivity and high-specificity has always been expected for clinical testing, yet, due to the lack of uniform detection methods and standards, the validity, reproducibility, and generality of the exosome research results are still questionable. Therefore, the comparability, stability, and validity of exosome detection results will become a major challenge for its future application.

Luckily, aptamers are suitable for the production of biosensors, biological recognition, and signal transduction. Their flexible combination with scientific technologies to amply fluorescence signals and transfer energy has greatly increased exosome detection sensitivity, improved the efficiency of disease diagnosis and monitoring, and provided a bright future for POC analysis. With more future studies on the analysis of exosome surface components, the accuracy of both exosome detection and disease diagnosis will be improved.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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