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REVIEW

Biosensor-based assay of exosome biomarker for early diagnosis of cancer

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Abstract **Cancer imposes a severe threat to people's health and lives, thus pressing a huge medical and economic burden on individuals and communities. Therefore, early diagnosis of cancer is indispensable in the timely prevention and effective treatment for patients. Exosome has recently become an attractive cancer biomarker in noninvasive early diagnosis because of the unique physiology and pathology functions, which reflects remarkable information regarding the cancer microenvironment, and plays an important role in the occurrence and evolution of cancer. Meanwhile, biosensors have gained great attention for the detection of exosomes due to their superior properties, such as convenient operation, real-time readout, high sensitivity, and remarkable specificity, suggesting promising biomedical applications in the early diagnosis of cancer. In this review, the latest advances of biosensors regarding the assay of exosomes were summarized, and the superiorities of exosomes as markers for the early diagnosis of cancer were evaluated. Moreover, the recent challenges and further opportunities of developing effective biosensors for the early diagnosis of cancer were discussed.**

Keywords biosensor; exosome; cancer diagnosis

Introduction

Cancer, an urgent global public health issue, constantly threats all human beings, and the survival rate of patients still could not be greatly improved over the past few years, partly due to the late discovery of cancer [1]. Therefore, the progressive development of early diagnosis and treatment of cancer has brought dawn for human beings to overcome cancer [2]. However, traditional cancer diagnostic methods not only are expensive but also require advanced instruments and professional medical staff, especially some methods that need invasive sampling [3]. Therefore, an urgent need for non-invasive, cost-effective, and easy-operation methods for tumor diagnosis exists. At present, the biomarker-based liquid biopsy is a hotspot in the early diagnosis of cancer; it has the salient characteristics of minimally invasive and repeated sampling. It could also comprehensively reflect the state information of tumor cell state [2,4] and pave the way for precision treatment and personalized medicine [5]. Thus far, several novel biomarkers specific to various cancers in fluid circulation have been found and played important roles in cancer diagnosis, especially exosome [6,7], of which superiorities lie in comprehensive information, high content in biofluids (1×10^9 particles/mL in the blood), abundant cargos, stable circulation, easy capture, and enrichment of surface proteins.

Exosomes contain many kinds of contents, such as nucleic acids [8], lipids [9], and proteins [10], which all play critical roles in performing special biological functions [11,12]. Various studies have revealed that exosomes are associated with the pathogenesis and progress of cancer [13–15]. Moreover, cancer cells may secrete more cancer-related exosomes than normal cells [16]. Therefore, detecting and monitoring exosomes and their contents could be a promising approach to screen cancer in liquid biopsy [17].

Researchers have investigated many methods to detect exosomes, including Western blotting (WB), ELISA [18], nanoparticle tracking analysis (NTA) [19,20], flow cytometry [21], and mass spectrometry [22]. Although these conventional quantitative methods are widely used, they have some shortcomings and limitations. For example, NTA could determine exosome purity, concentration, and size distribution, but it could not distinguish the phenotype within complicated samples [20,23]. Although ELISA and

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WB could identify the protein information of exosomes, they are unable to quantify the concentration [24]. Besides, the promotion of these methods is dragged down by the problems of cumbersome operation, complicated sample pretreatment, and expensive analytical instruments [25–27]. Identifying the exosomes in body fluids with fast and cost-effective approaches is urgent for the early diagnosis of patients with cancer [28], especially those who lived in resource-poor regions [29].

A biosensor is a sophisticated system applied to detect a specific biological analyte by converting bio-recognition events into measurable signals. Biosensors are nowadays ubiquitous in many areas, especially in biomedical research, disease diagnosis [30], food safety [31], and drug discovery [32]. As a promising candidate for conquering the challenges at this stage, biosensors could boost the field of liquid biopsy [33,34] owing to their small size, fast response, and high sensitivity [6,35]. In the past years, researchers have proposed a large number of biosensors for exosome assays with extraordinary performance and then applied them in medical and biological fields [34,36]. The advancement in clinical molecular diagnosis enables enhanced prediction of clinical outcome, increased reasonable patient stratification, and enhanced treatment decision-making, which has brought substantial benefits to patients [37].

Several reviews that refer to exosome detection have been recently published [28,35,38]. The present article focused on the superiorities of exosomes acting as a biomarker in cancer diagnosis, and various types of biosensors, including electrochemical biosensor, colorimetric biosensors, fluorescence-based biosensors, surface plasmon resonance (SPR)-based biosensors, surface-enhanced Raman scattering (SERS)-based biosensors, and microfluidic-based biosensors, were comprehensively summarized, with an emphasis on their analytical performances and further challenges in the clinic diagnosis of cancer and the potential solutions.

Exosome biology, role, and function

Exosomes are a kind of biological extracellular vehicles secreted by cells and coated with bilateral phospholipid membranes [12]. These vesicles, with a diameter of 60–100 nm, circulate in almost all kinds of body fluids [39] and serve as messengers in the intercellular communication. They are also associated with extracellular matrix remodeling [14,40]. The definition of exosome changes with increasing investigation. The description of small vesicles in biological fluids could be traced back to the late 1960s, when they were considered as a waste released by platelets [41]. In 1985, Johnstone *et al*. discovered a kind of small vesicles in the multivesicular bodies of reticulocytes that could be released to extracellular surroundings, and then termed this small vesicle as “exosome” [42]. Thereafter, these unique vesicles have been found in various body fluids, such as blood, saliva, semen, amniotic fluid, ascites, urine, cerebrospinal fluid, breast milk, and bile [43,44].

Exosomes contain thousands of proteins, nucleic acids, metabolites, and lipids, which could all be transferred into cells and affect specific physiologic and pathological processes in organisms. They could participate in mammalian reproduction and development [44,45], immune responses [46], bacterial infection [47], and viral infection [48]. They are also involved in the metabolic process [49], the protection of cardiovascular fitness [50], and neurodegeneration modulation [51]. Exosomes are related to cancer origin and development [52]. In particular, exosomes could modulate immune surveillance [53] and promote cell migration [54], invasion [55], epithelial–mesenchymal transition [14,15], and phenotypic transformation [56].

The abundant biological molecules in exosomes also play important roles in disease diagnosis. For example, the proteins of exosomes reflecting the biological information of their parent cells could affect the functions and physiology of recipient cells. Therefore, exosomal proteins have been used as tools for diagnosis, especially in cancers, such as lung cancer [57], pancreatic cancer [58], gastric cancer [59], colorectal cancer (CRC) [16], breast cancer [60], and ovarian cancer [61] (Table 1). It is notable that exosomal surface proteins could serve as surface markers to facilitate the enrichment of cancer cell-derived exosomes. Exosomes also contain several nucleic acids. For instance, miRNAs could circulate stably in serum and enter into the adjacent cells, affecting the gene expression of recipient cells [62]. Meanwhile, the composition and amount of exosomal miRNAs in normal cells differ from those in cancer cells, suggesting their potential in the early diagnosis of cancer [63]. Thus far, several exosomal miRNAs have been deemed as biomarkers in diverse cancers (Table 2), such as prostate cancer [64], cervical carcinoma [65], and pancreatic cancer [66]. Exosomal contents may provide significant and specific information regarding the tumor microenvironment. Therefore, exosomes, which are involved in a series of physiology and pathology processes, have become a star in clinical study at present [67]. Their applications in the early diagnosis of cancer are increasing considerably [68].

Electrochemical biosensors

Electrochemical biosensors have been applied in exosome detection because of their advantages, such as good adaptability, high sensitivity, facile operation, great convenience, fast, and low cost, making them quickly adapt to clinical settings [75,76]. An electrochemical biosensor is

Table 1 Exosomal proteins as tumor biomarkers

Exosomal proteins	Pathophysiology	Source	Isolation method	Year	References
CD91, CD317, and EGFR	Lung cancer	Plasma	Extracellular vehicles array	2015	[57]
LRG1	Lung cancer	Urine	Ultracentrifugation	2011	[69]
NY-ESO-1	Lung cancer	Plasma	Extracellular vehicles array	2016	[10]
LESP-1	Lung cancer	Plasma	Exclusion chromatography	2020	[70]
GPC1	Pancreatic cancer	Serum	Flow cytometry	2015	[58]
GKN1	Gastric cancer	Serum	Ultracentrifugation	2018	[59]
CEA	Colorectal cancer	Serum	Ultracentrifugation	2012	[16]
CD147	Colorectal cancer	Plasma	Ultracentrifugation	2018	[71]
Survivin-2B	Breast cancer	Serum	Ultracentrifugation	2014	[72]
FRα	Ovarian cancer	Plasma	Microfluidic chip	2019	[61]

Table 2 Exosomal miRNAs as tumor biomarkers

Exosomal miRNAs	Pathophysiology	Source	Isolation method	Year	References
miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205, and miR-214	Ovarian cancer	Plasma	Modified MACS[a]	2008	[73]
miR-19b-3p, miR-584-5p, miR-425-5p, miR-221-3p, miR-409-3p, and miR-21-5p	Lung adenocarcinoma	Plasma	ExoQuick	2016	[74]
miR-141	Prostate cancer	Serum	ExoQuick	2015	[64]
miR-221-3p	Cervical carcinoma	Serum	ExoQuick	2019	[65]
miR-301a-3p	Pancreatic cancer	Serum	Ultracentrifugation	2018	[66]

[a]MACS, magnetic activated cell sorting.

mainly composed of two parts: a bio-recognition element and a transducer. After the exosomes in test samples are captured by specific bio-recognition elements, the transducers could translate the recognition event into electrochemical signals (amperometry, potentiometry, conductometry, and impedimetry). For the development of electrochemical biosensors, nucleic acid is a frequently-used element in the fabrication of high-sensitivity electrochemical biosensor because of its ultra-high efficiency, base-pairing precision, and low cost [77]. Moreover, aptamer, which shows a strong affinity to specific proteins, has been widely used in the design of label-free electrochemical strategies for exosome detection [76]. Yin *et al*. proposed an aptamer-triggered, simple, and sensitive method by embedding doxorubicin into double-strand DNA to serve as electrochemical signal reporters. After the aptamer-triggered method was combined with exonuclease III (Exo III)-assisted signal amplification, the limit of detection (LOD) was down to 12 particles/μL [78]. Similarly, Dong *et al*. used the recognition capacity of aptamers to release several messenger DNAs and exploited Exo III to trigger the next round of signal amplification. The dual-signal amplification assay could determine targets as low as 70 particles/μL [79]. Interestingly, by the virtue of two aptamers that could simultaneously bind to two proteins that coexist on the same exosome, Zhao *et al*. developed an ultrasensitive approach for exosome detection by using a target-triggered 3D DNA walking machine. The biosensor exhibited great analytical performance and obtained a detection limit of 14 particles/μL under optimal conditions [80]. Wang *et al*. implemented a multidirectional hybridization chain reaction (HCR)-based electrochemical aptasensor to detect MCF-7 cell-derived EpCAM positive exosomes [81]. They used hydrophobic interaction between cholesterol anchor and exosomal lipid bilayer to trigger multidirectional HCR. This biosensor exhibited high sensitivity with an LOD of 285 particles/μL. Click chemistry has also been used for the design of biosensors. For instance, An *et al*. combined click chemistry and HCR reaction with a glassy carbon electrode for exosome measurements [82]. Instead of exploiting cholesterol to introduce the primer of HCR, this method was based on nonspecific capture (4-oxo-2-nonenal alkyne (alkynyl-4-ONE)), which could enhance the accuracy and sensitivity, with a low LOD of 96 particles/μL. Moreover, by introducing a DNA molecule machine in building a biosensor, Cao *et al*. proposed an amplified electrochemical detection method for exosome assay. The cascade toehold-mediated strand displacement reaction could continually introduce methylene blue (MB)-modified

signal probes to generate enhanced signals [83]. Besides, to further improve the selectivity and efficiency of exosome assay for the early diagnosis of cancer, Huang *et al*. screened a gastric cancer exosome-specific aptamer towards Mucin 1 (MUC1), which could serve as a potential biomarker to predict the invasion of gastric cancer [84]. By further making use of rolling circle amplification (RCA) reaction, they acquired a low detection limit of 954 particles/mL [85]. Though the abovementioned nucleic acid-based signal amplification methods showed the superiorities of high sensitivity, high specificity, and low cost, they may suffer from relatively laborious sequence design. Wang *et al*. recently devised a spherical nucleic acid-based cascade signal amplification strategy for exosome detection. By the virtue of terminal deoxynucleotidyl transferase (TdT) and Exo III, a convenient-operated and highly sensitive method for the detection of exosomes without complicated nucleic acids sequence design was proposed, acquiring a low LOD of 44 particles/μL [86].

In addition to DNA-based amplification, some nanomaterials have been used to improve the performance of biosensors [87]. For example, metal–organic frameworks (MOFs) have been explored for the fabrication of electrochemical biosensor owing to their excellent chemical tenability [88]. Sun *et al*. (Fig. 1A) designed an enzyme-free and label-free electrochemical biosensor by exploiting zirconium-based MOFs (Zr-MOFs) to sensitively detect glioblastoma-derived (GBM) exosomes [25]. In this ingenious design, the Zr-MOFs loaded with methylene blue molecules (MB@Zr-MOFs) could directly combine to GBM exosomes via the formation of Zr-O-P bonds [89]. When the GBM exosomes were selectively captured by a peptide ligand [90], the MB encapsulated in Zr-MOFs generated a high electrochemical signal. Consequently, the detection could be obtained from 9.5×10^3 particles/μL to 1.9×10^7 particles/μL, and the LOD was 7.83×10^3 particles/μL. The proposed strategy showed good versatility and effectiveness as it could be used to detect other disease biomarkers by simply replacing the capture sequences. MOFs could encapsulate diverse biological molecules due to the adjustable porosity [91] and release the contents in response to different endogenous or exogenous stimuli. Cao *et al*. (Fig. 1B) utilized a kind of pH-responsive MOFs, zeolitic imidazolate framework-8, to pack horseradish peroxidase (HRP) and developed an electrochemical biosensor for accurate identification of PD-L1$^+$ exosomes in breast cancer [92]. By combining DNA amplification-controlled pH decreases with the virtue of MOF-mediated signal amplification, the obtained results of the biosensor showed an LOD of as low as 334 particles/mL and a linear range of 1×10^3–1×10^{10} particles/mL. Covalent organic frameworks (COFs) have also been applied in sensor fabrication due to their admirable loading capacity, ordered crystal structure, and decent biocompatibility [93]. Wang *et al*. (Fig. 1C) designed an HRP-pSC_4-AuNPs@COFs nanoprobe for the detection of CRC-derived exosomes [94]. In the presence of TMB and H_2O_2, the COFs endowed HRPs with high catalytic activity and produced an enhanced electrochemical signal. This biosensor showed excellent analytical performance, and it could measure exosomes in the range of 5×10^2–5×10^7 particles/μL, with an LOD of 160 particles/μL. Some nanoparticles with peroxidase-mimicking activity could also be used to build electrochemical biosensors. Shiddiky *et al*. recently utilized carboxyl group-functionalized iron oxide (Fe_2O_3) nanoparticles (C-IONPs) for direct isolation and quantification of exosomes. By the virtue of carboxylic acid –COOH groups on the surface, the C-IONPs could be conjugated to CD9 and thus served as capture agents for exosome separation and purification. The selected nanomaterials could be used as dispersible nanocarriers and nanozymes, which realized the integration of isolation and detection [95].

Table 3 summarizes the abovementioned biosensors' performance. Electrochemical biosensors could be an undisputed useful technology for exosome assay due to their high sensitivity, rapid response, and cost-effective system [35,96].

Table 3 Electrochemical biosensor for exosome detection

Detection method	Target	Type of cancer	Sample	Recognition element	Signal element	LOD	References
Electrochemistry	EGFRvIII/EGFR	Glioblastoma	Serum	Peptide	MB	7.83×10^3 particles/μL	[25]
Electrochemistry	CD63	Breast cancer	Serum	Aptamer	HRP-OPD	96 particles/μL	[82]
Electrochemistry	EpCAM	Breast cancer	Serum	Aptamer	HRP-H_2O_2-TMB	285 particles/μL	[81]
Electrochemistry	MUC1	Gastric cancer	Plasma	Aptamer/antibody	G-quadruplex/H_2O_2	954 particles/mL	[85]
Electrochemistry	EpCAM	Colorectal cancer	Plasma	Aptamer	MB	44 particles/μL	[86]
Electrochemistry	PD-L1/CD63	Breast cancer	Serum	Aptamer/antibody	HRP-OPD	334 particles/mL	[92]
Electrochemistry	CD63	Colorectal cancer	Serum	Aptamer	HRP-H_2O_2-TMB	160 particles/μL	[94]
Electrochemistry	CD9/CA-125	Ovarian cancer	–	Antibody	Fe_2O_3-H_2O_2-TMB	1.25×10^6 exosomes/mL	[95]

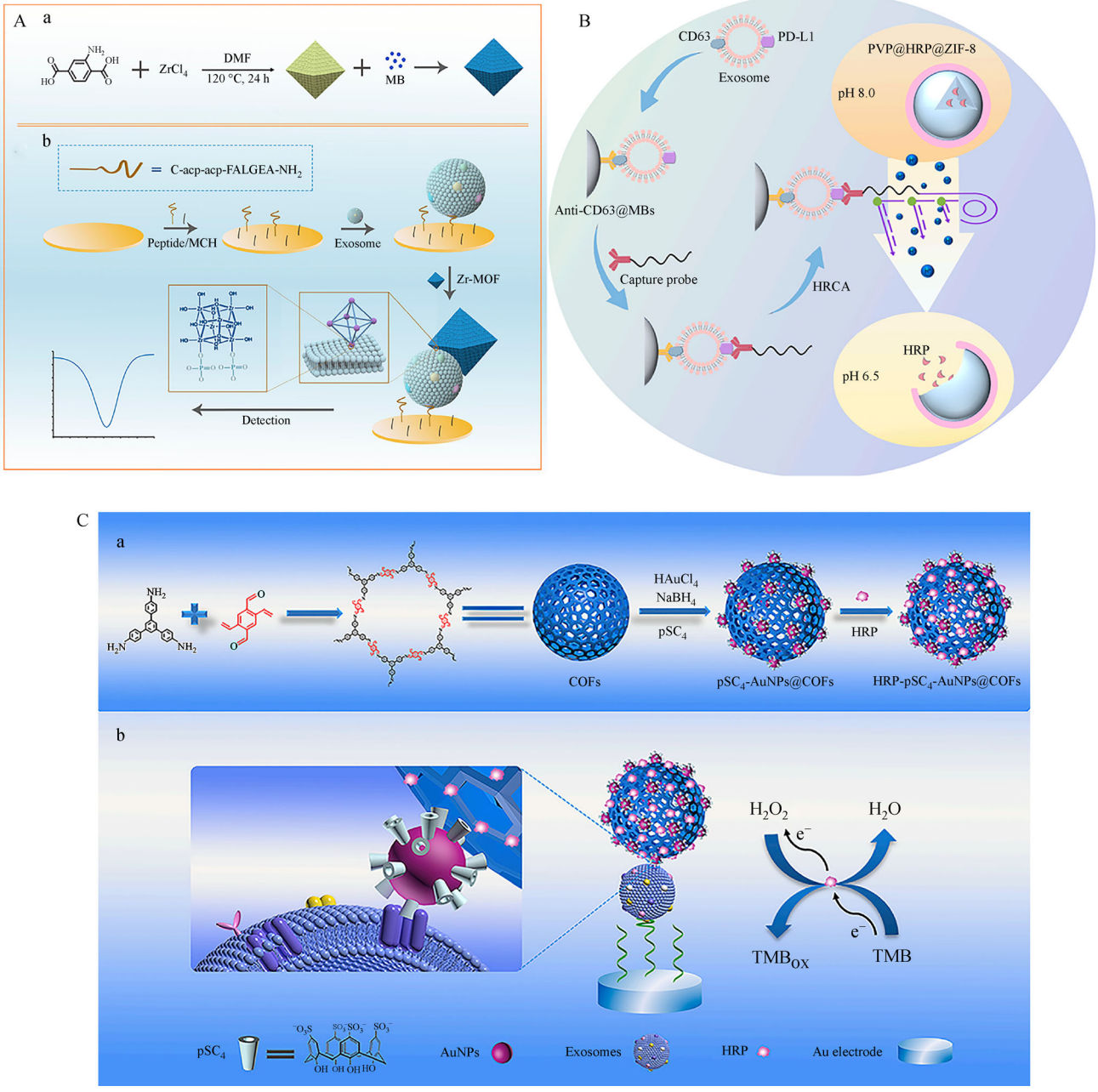

Fig. 1 Schematic of representative electrochemistry-based biosensors. (A) Detection of circulating exosomes by using zirconium-based metal-organic frameworks (Zr-MOFs). (a) Fabrication process of Zr-MOFs and Zr-MOFs loaded with methylene blue (MB@Zr-MOFs). (b) Process of electrochemical biosensor for exosome detection using MB@Zr-MOFs. (Reproduced with permission from Ref. [25]. Copyright 2020 American Chemical Society.) (B) Ultrasensitive electrochemical biosensor facilitated with DNA amplification-controlled pH decreases based on pH-responsive MOFs encapsulated with horseradish peroxidase (HRP). (Reproduced with permission from Ref. [92]. Copyright 2020 Elsevier.) (C) Novel nanoprobe-based electrochemical assay for colorectal cancer-derived exosomes. (a) Fabrication process of covalent organic frameworks (COFs)-based nanoprobes. (b) Process of exosomes detection by using HRP-pSC4-AuNPs@COFs nanoprobe. (Reproduced with permission from Ref. [94]. Copyright 2020 Elsevier.)

Fluorescence-based biosensors

Fluorescence-based methods have been widely used in biosensor development because of their simple operation, outstanding sensitivity, and cost effectiveness [97]. The three main methods for biosensor fabrication are fluorescence-based signal amplification, fluorescence resonance energy transfer (FRET), and fluorescence polarization. As an effective signal readout tool, fluorescence could be combined with various signal amplification strategies to

fabricate sensitive biosensors for exosome detection [98]. Li and coworkers proposed several methods to use liposomes as an intriguing amplification element. For example, they used Zr^{4+} to serve as a bridge to link exosomes and liposomes due to the intrinsic combination of Zr^{4+} and the phosphate group outside vesicles. By the virtue of the interaction, exosomes could be detected conveniently without complicated operations [99]. The researchers also took advantage of aptamer-coated cationic liposome to further enhance the analytical performance [26]. In this work (Fig. 2A), TdT assisted with thioflavin T (ThT) were used to amplify the fluorescence signals. The LOD of this biosensor was 360 particles/μL, more sensitive than that in a previous report [100]. In addition, the researchers utilized the steric hindrance effect to control signal amplification and designed a simple and sensitive fluorescent biosensor (Fig. 2B). The attempt to detect exosomes on the account of its intrinsic steric hindrance effect was successful [101]. These studies have made full use of the own characteristics of exosomes, and they may bring more inspiration in the future for the construction of biosensors.

RCA is of great significance among nucleic acid-based signal amplification techniques, because it generates long-strand DNA at room temperature [102]. In a work performed by Huang *et al.* (Fig. 2C), an RCA reaction was introduced to generate many repeat sections for hybridization with a nanoprobe composed of gold nanoparticle (GNP)-DNA-fluorescent dye (FAM, GNP-DNA-FAM). With the digestion of nicking endonuclease (Nb·BbvCI), fluorescence signals continuously accumulated, and an LOD for 100 particles/μL of exosome detection could be obtained [103]. Instead of the help of endonuclease to amplify the readout signal, a second primer was recently introduced to trigger a branched RCA to construct fluorescent biosensor; thus, the cost was cheaper and the operation was simpler than before, but the sensitivity was slightly weakened [104].

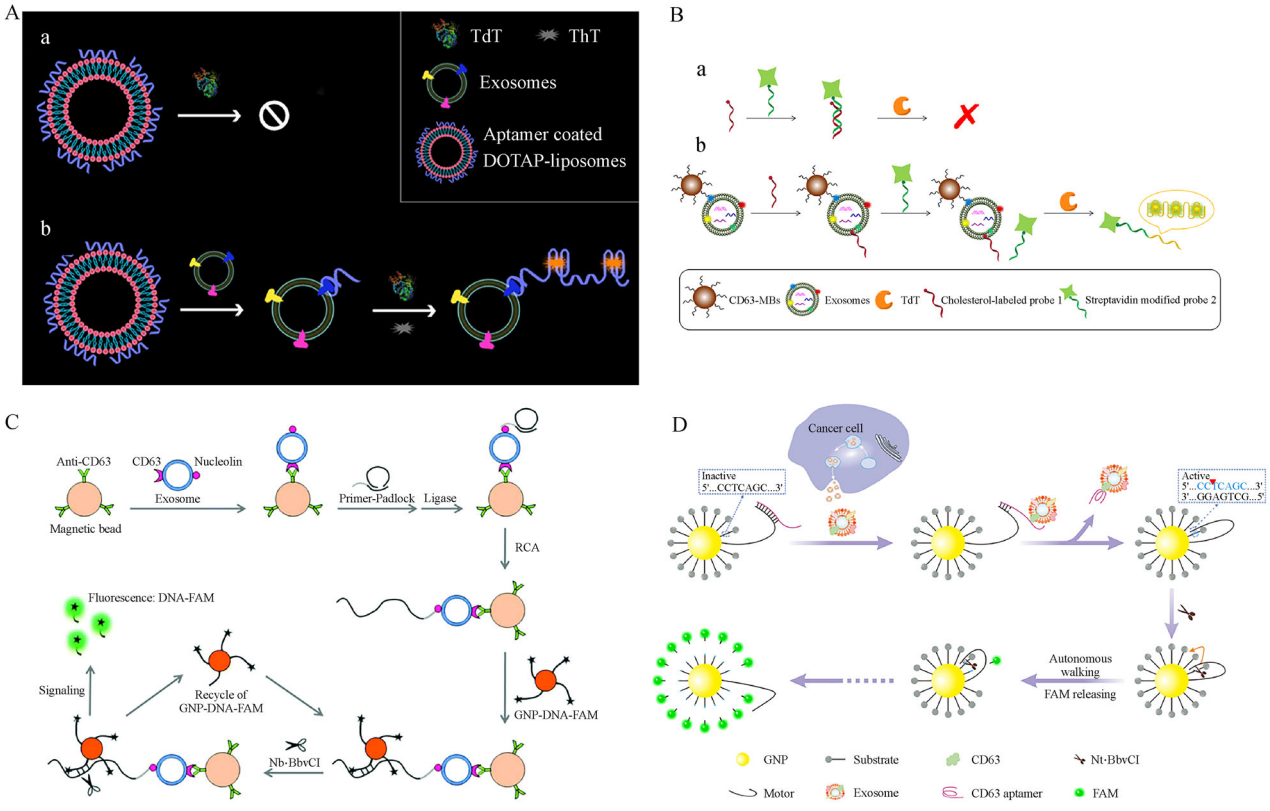

Fig. 2 Schematic of representative fluorescence-based biosensors. (A) Fabricating a cationic liposome for signal amplification detection of exosomes. Only in the presence of exosomes, the electrostatic attraction between aptamers and liposome broke down and then the free aptamers could trigger enzyme-mediated DNA extension to form multiple G-quadruplex. (Reproduced with permission from Ref. [26]. Copyright 2019 American Chemical Society.) (B) Simple and sensitive strategy for exosome detection utilizing steric hindrance-to-control signal amplification. (Adapted from Ref. [101], with permission from the Royal Society of Chemistry.) (C) Dual-signal amplification constructed using GNP-DNA-FAM conjugates hybridized with RCA technique (Adapted from Ref. [103], with permission from the Royal Society of Chemistry.) (D) Construction of exosome-triggered enzyme-powered DNA motors for sensitive exosome assay. (Reproduced with permission from Ref. [107]. Copyright 2020 Elsevier.)

Concerning the fluorescent biosensors based on the FRET principle, Zhang *et al*. designed a "turn on" fluorescent aptasensor by selecting tetramethylrhodamine (TAMRA) as luminophore and Dabcyl as quenching group. Exosomes could break the hairpin structure of probes, leaving the TAMRA far away from the quencher, and then a strong fluorescence could be detected [105]. Li *et al*. combined another "fluorophore-quencher pair" (quencher (BHQ) and fluorescent dye (FITC)) with a superparamagnetic conjunction to construct a dual functional biosensor for the detection and capturing of exosomes from prostate cancer. Excellent diagnostic efficiency could be achieved with the elaborate design [106]. Yu *et al*. proposed a three-dimensional (3D) DNA motor-based biosensor for exosome assay (Fig. 2D). In this design, GNP was modified with carboxyl fluorescein-labeled DNA strands to build DNA motors, which were locked with aptamer strands. Exosomes competitively bound to aptamers and triggered the DNA motor process, while restriction endonuclease Nt·BbvCI continually powered the FAM-labeled segment release. Compared with that in conventional ELISA, the LOD was fairly low (8.2 particles/μL) [107]. Li *et al*. constructed a facile fluorescent aptasensor, which was composed of aptamer, positively charged tertiary amine-containing tetraphenylethene (TPE-TA), and graphene oxide (GO), as recognition elements, fluorescent dye, and quencher, respectively. Numerous TPE-TAs bound one aptamer and aggregated together to produce amplified fluorescence signals in the presence of exosomes; otherwise, the signals were quenched by GO [108]. In a similar principle, Wang *et al*. used GO to absorb and quench fluorophore. With the assistance from an enzyme, an enlarged signal could be gained [47].

Through aptamer-based fluorescence polarization, Zhang *et al*. developed a simple, direct, and sensitive approach for exosome quantification in human plasma. Owing to the inherently huge mass/volume of exosomes, they could act as mass-based fluorescence polarization amplifiers to realize sensitivity detection. The assay achieved an LOD of 500 particles/μL for lung cancer-derived exosomes [109]. By simple mix-and-read, this method could detect exosomes in the biological matrix within approximately half an hour, without redundant separation or amplification procedures.

Colorimetric biosensors

Colorimetry exhibits attractive potential in biosensor construction due to its cost effectiveness, visual output, and simplicity. Colorimetric biosensors could transform the detection events into apparent color changes through two main approaches: distances or dispersion status change in nanomaterials and oxidation reactions mediated by enzymes [110,111]. Thus, nanomaterials and enzymes are commonly used as signal tags in colorimetric assay [112]. Given that the color changes in GNPs resulted from aggregation, Liu *et al*. proposed a colorimetric biosensor to detect exosomes through the combination of recombinase polymerase amplification with transcription-mediated amplification. They quantified the amplified surrogate DNA signal for exosome via GNP-based colorimetric assay. This design obtained a low LOD of 102 particle/mL and thus may have a great potential in the early diagnosis of nasopharyngeal carcinoma [113]. Oxidation reactions could also be applied in colorimetric assay as the result of the color reaction between enzyme and substrate. For example, HRP could extensively accelerate the polymerization of colorless dopamine to produce a brown-black colored polydopamine (PDA) [114]. In accordance with this finding, Xu *et al*. (Fig. 3A) fabricated a colorimetric biosensor by introducing HRP to the surface of the exosomes and realized the detection within 10 min [111]. The sensitivity was further enhanced because the formed PDA could deposit around exosomes via the outstanding reactivity between PDA and amine, sulfhydryl, and the phenol groups of proteins [115]. The simple and cost-effective colorimetric biosensor with an LOD of 7.77×10^3 particle/mL deserved to be further studied to focus on the validation of clinical patient samples and the early detection of cancer. TMB is another common substrate of HRP. Chen *et al*. constructed a visible and colorimetric biosensor for the detection of exosomes by combining HRP catalysis and a ZnO nanowire-based 3D scaffold chip. Owing to the large surface area of ZnO-chip device, the biosensor acquired an improved capture efficiency for exosome assay and an LOD of 2.2×10^4 particles/μL [116]. He *et al*. also used TMB as the chromophore substrate to react with HRP for exosome detection. In this work, targets were first enriched by immunomagnetic beads and then inserted with a bivalent-cholesterol-labeled DNA to trigger the HRP-linked HCR for color reaction. The DNA nanostructure could load many HRPs for signal amplification, making the LOD down to 2.2×10^3 particles/μL [117].

Some enzymes could be specifically used for the fabrication of colorimetric biosensors. For instance, Zhang *et al*. applied alkaline phosphatase (ALP) in the construction of biosensors [118,119]. They proposed a visual colorimetric method via ALP-induced silver deposition on gold nanorods (Au NRs) (Fig. 3B). An HCR process was involved here to accumulate ALP, which realized the multicolor visual detection of exosomes [118]. The operation was kind of complex, but the LOD could be as low as 1.6×10^2 particles/μL.

Although enzyme catalysis is one of the most common methods employed for the development of colorimetric biosensors, natural enzymes are complicated to purify and easily deactivated under harsh conditions, which may limit

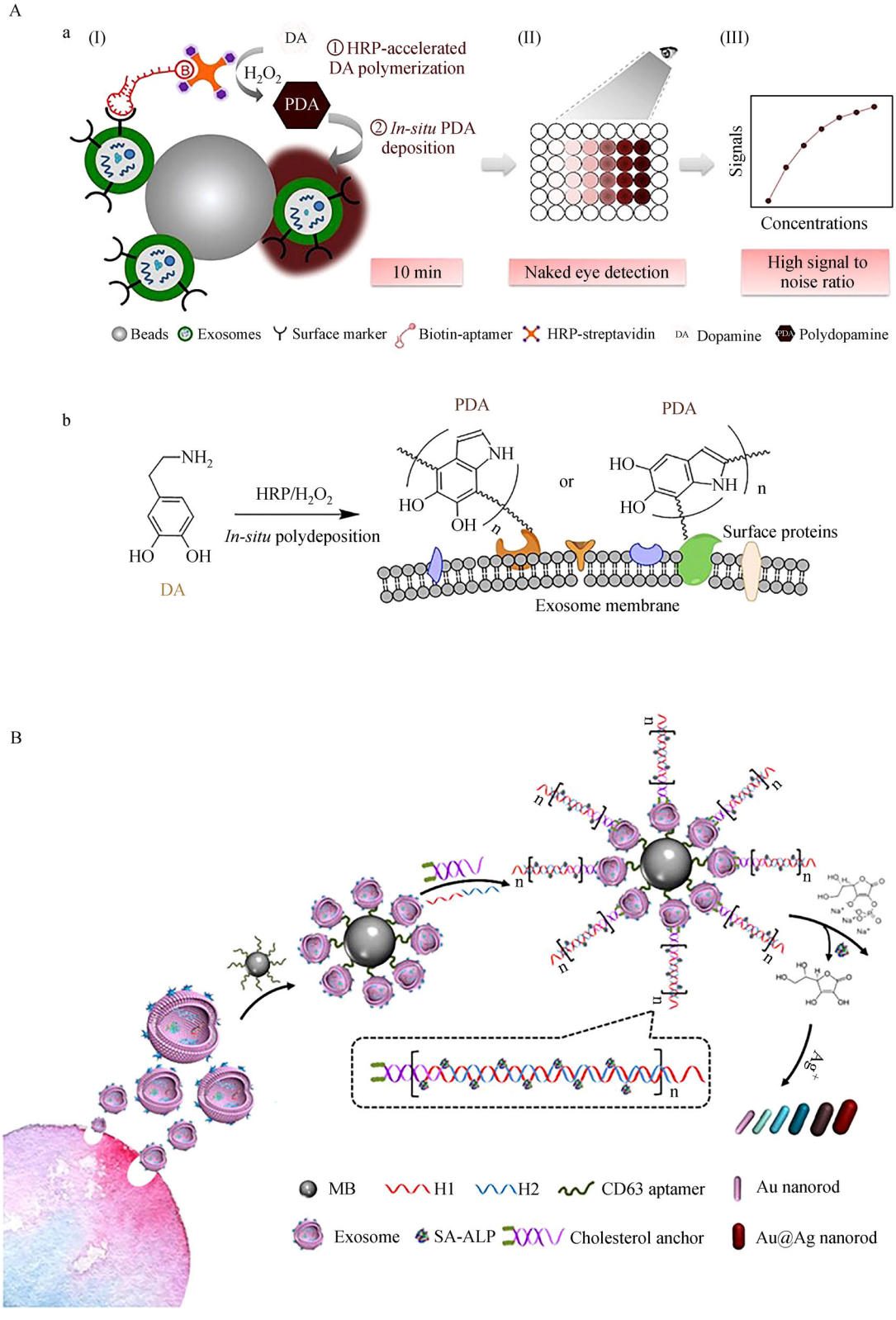

Fig. 3 Schematic of representative colorimetry-based biosensors. (A) Enzymatic catalysis-based dopamine polymerization and *in-situ* deposition for the design of colorimetric aptasensor for exosome detection. (a) Capturing target exosomes with latex beads and then using biotin-labeled aptamers to introduce streptavidin-labeled HRP to accelerate the polymerization of dopamine. A distinguishable color change could be seen, which could be strengthened by *in-situ* deposition of polydopamine around exosome particles. (b) Process of dopamine polymerization and polydopamine deposition on the surface of exosomes. Polydopamine could link to amine, sulfhydryl, and the phenol groups of proteins. (Reproduced with permission from Ref. [111]. Copyright 2020 Elsevier.) (B) Sensitive multicolor visual biosensor using a dual-signal amplification strategy of enzyme-catalyzed metallization of Au nanorods and hybridization chain reaction. (Reproduced with permission from Ref. [118]. Copyright 2019 American Chemical Society.)

their application. Nanozymes with high chemical and thermal stability recently gained considerable attention in the construction of biosensors [120]. For example, Fe_3O_4 and Fe_2O_3 nanoparticles have high peroxidase activity; they could catalyze TMB from colorless to blue in the presence of H_2O_2. Therefore, by functionalization of iron oxide, aptamer-capped Fe_3O_4 NPs [121] and gold-loaded ferric oxide nanocubes [122] have been used for the fabrication of a colorimetric biosensor to detect exosome. Wang *et al*. used g-C_3N_4 NSs as nanozymes to build a "signal-off" colorimetric biosensor for exosome analysis. When exosomes despoiled the aptamers from the nanosheets, color change could be observed because of the decrease in the catalytic ability of nanozymes [123]. The peroxidase activity of single-walled carbon nanotubes was employed via aptamer absorption to develop an aptamer-based colorimetric biosensor [124]. This proposed method could be universally applicable for diverse exosome detection by simply changing the aptamer sequences. Moreover, hemin/G-quadruplex complex could catalyze the H_2O_2-mediated oxidation of TMB and produce an obvious color change; thus, it has also been used to realize colorimetric detection of exosomes [125].

SPR-based biosensors

SPR-based biosensors could detect molecular interactions by monitoring the refractive index changes on the surface of a thin gold layer, and they have been widely applied in biomolecule analysis in a real-time and label-free manner [126]. Many biological receptors, such as antibody, aptamer, and enzyme, have been employed to form SPR biosensors on the basis of distinctive optical phenomenon [127]. Moreover, SPR is highly sensitive to the binding events among the range of 200 nm on the gold layer [128]. This size closely fits with the dimension of exosomes. Thus, SPR-based biosensors have natural advantages for the assay of exosomes. Wang and coworkers designed an aptamer-based SPR biosensor for the highly sensitive detection of exosomes on the basis of dual GNP-assisted signal amplification [129]. In the presence of exosomes, a single AuNP-amplified SPR signal could be achieved through the complementary base hybridization (Fig. 4A). This enzyme-free method may reduce constraints on the detection conditions and offer opportunities for the development of exosome-based disease diagnostics. Unlike using GNPs for SPR signal enhancement, Wu *et al*. reported a localized surface plasmon resonance (LSPR) biosensor with self-assembly gold nanoislands for the detection and discrimination of different extracellular vesicles (A-549, SH-SY5Y) [130]. Later, they developed biotinylated antibody-functionalized titanium nitride (BAF-TiN) biosensor for high-performance label-free detection of human glioma cell line-derived exosomes (Fig. 4B) [131]. In this biosensor, they used TiN as alternative plasmonic supporting material rather than gold and silver substrate, showing tunable plasmonic properties in the visible and near-infrared spectra. Meanwhile, biotinylated anti-EGFRvIII antibody could be immobilized on TiN surface on the basis of high-affinity biotin-TiN interaction, which could solve the problem of surface functionalization to TiN. The proposed BAF-TiN structure also exhibited excellent sensitivity and selectivity towards GM-derived exosomes. The latter work acquired higher accuracy than the previous one of the researchers, and it could have a great potential for exosomal surface protein analysis. For multiplex analysis of different exosome proteins, Im *et al*. reported a high-throughput, label-free nano-plasmonic exosome assay for quantitative analysis of exosomes on the basis of transmission SPR through periodic nanohole arrays [132]. Antibody-functionalized array could profile the exosome surface proteins with improved sensitivity over other methods. It integrated with miniaturized optics to enable portable operation. This biosensor could identify ovarian cancer cell-derived exosomes through analysis of CD24 and EpCAM membrane proteins. Due to the characteristics of label-free and real-time measurement, SPR-based biosensors have been rapidly developed and made great progress for the detection of exosomes. However, most of SPR-biosensors are employed only for purified exosome samples prepared via ultracentrifugation, which may cause the detection process to be time consuming. Another limitation of SPR biosensor is the inability for multiplex analysis of exosomes.

SERS-based biosensors

For the detection of low-amount biomarkers, Raman scattering or SERS is a powerful plasmonic technique that is also a popular optical method because of its remarkable potential to enhance signals. A SERS-based biosensor is mainly composed of two parts: a substrate fixed with recognition elements and SERS nanoprobes modified with signal elements, and a sensor system that could form a typical sandwich in the presence of the target. SERS-based biosensors could identify distinctive spectral signals in the changeable and complicated biological environment compared with SPR or fluorescence [133]. It has been applied for the analysis of biomolecules, cellular imaging, and environmental monitoring [134].

SERS-based biosensors have also been employed to detect exosomes, with the advantages of rapid speed, high sensitivity, and multiplex analysis ability. Meanwhile, some nanomaterials have been used to improve the performance of these biosensors. Among them, Au nanomaterial is widely used for signal enhancement in SERS-based biosensors. Wang and coworkers proposed a

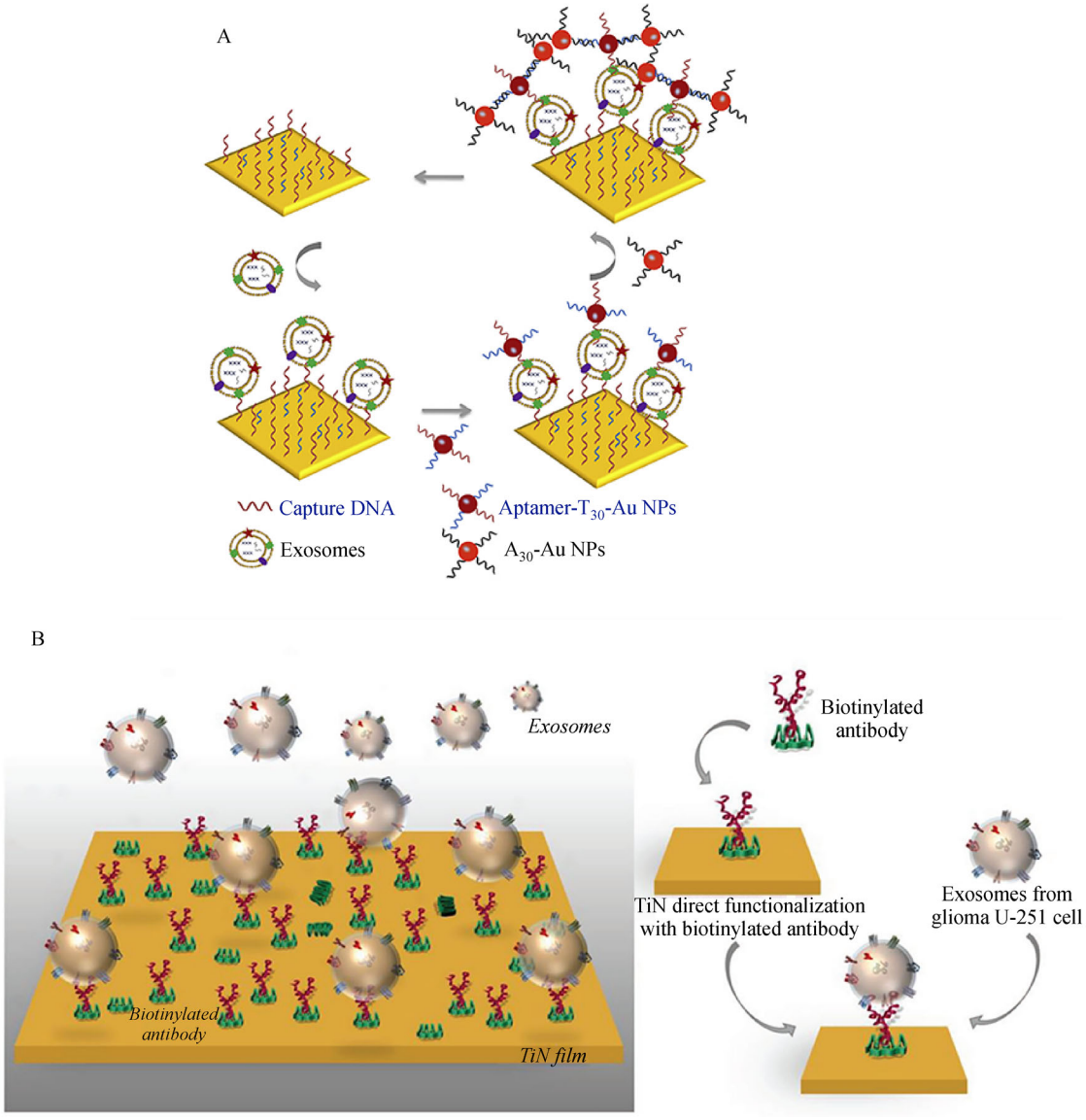

Fig. 4 Schematic of representative SPR-based biosensors. (A) Determination of cancerous exosomes based on dual AuNP-assisted signal amplification. (Reproduced with permission from Ref. [129]. Copyright 2019 Elsevier.) (B) Detection of glioma cells derived exosomes via biotinylated anti-CD63 antibody functionalized with titanium nitride (TiN). (Reproduced with permission from Ref. [131]. Copyright 2019 Wiley.)

SERS-based biosensor for simultaneous multiple detections of exosomes by using SERS probes (Fig. 5A) [135]. The SERS probe was prepared via GNP decorated with a Raman reporter and an aptamer for targeting a particular exosome. This biosensor could simultaneously detect multiple kinds of exosomes on the basis of three kinds of SERS probes designed using different SERS reporters. It could also distinguish the species of the target exosomes in one step. Zong *et al*. proposed a sandwich-type immunocomplex including SERS nanoprobes and magnetic nanobeads for the detection of tumor cell-derived exosomes (Fig. 5B) [136]. The SERS nanoprobe comprised gold core–silver shell NRs (Au@Ag NRs), Raman molecules, and a silica layer, which was decorated with antibody to specifically capture exosomes. SERS signals could be detected only in the presence of exosomes by forming sandwich-type immunocomplexes. The formation of GNPs could be varied in different strategies. For instance, Au NRs instead of spherical GNPs coated with QSY21 as SERS probes were used for quantitative detection of exosome proteins [137]. This designed device could analyze over 80 purified samples, with a detection limit of 2×10^6 exosomes/mL in 2 h. It required initial exosome isolation and could be further improved by

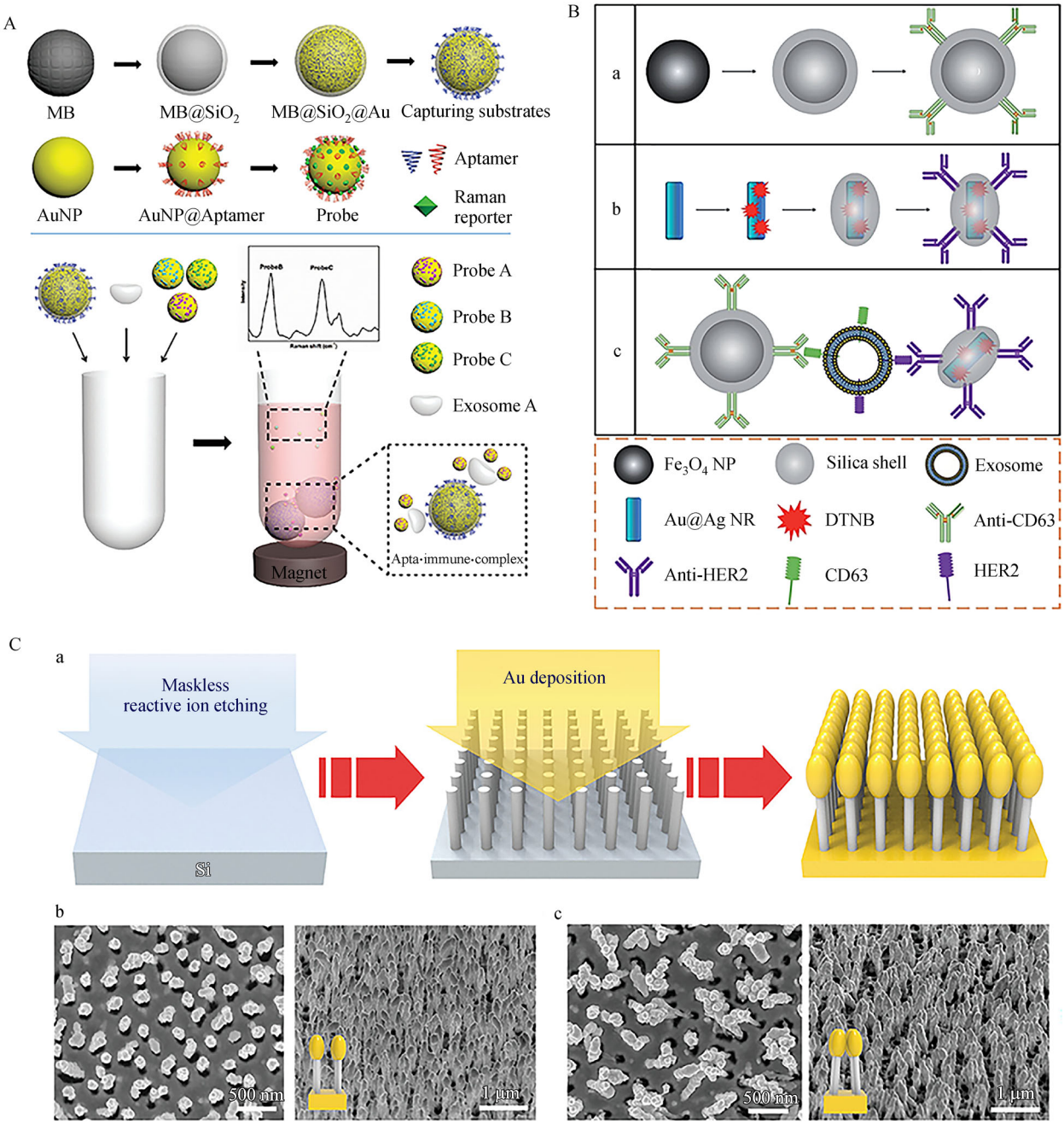

Fig. 5 Schematic of representative SERS-based biosensors. (A) Detection of cancer cells secreted exosomes based on Au nanoparticles decorated with a Raman reporter and magnetic beads with Au shell. (Reproduced with permission from Ref. [135]. Copyright 2018 the Royal Society of Chemistry.) (B) Detection of tumor-derived exosomes by using SERS nanoprobes with a core–shell structure and magnetic nanobeads. Diagram of the fabrication of (a) magnetic nanobead and (b) SERS nanoprobe. (c) Sandwich-type immunocomplex structure composed of exosome, magnetic nanobead, and SERS nanoprobe. Images are not to scale. (Reproduced with permission from Ref. [136]. Copyright 2016 the Royal Society of Chemistry.) (C) Detection of exosomal miRNAs based on plasmonic head-flocked gold nanopillars. (a) Fabrication process of plasmonic gold nanopillar SERS substrate. SEM images of (b) prepared plasmonic gold nanopillar SERS substrates and (c) plasmonic head-flocked gold nanopillar SERS substrates after solvent evaporation. (Reproduced with permission from Ref. [139]. Copyright 2019 Wiley.)

integrating with the optimal exosome isolation method.

Exosomal nucleic acids are also important for the diagnosis of tumor, and they have been studied by many researchers by using SERS-based assays. For example, Ma *et al*. reported a new SERS analysis method using stable SERS reporter element and duplex-specific nuclease-mediated signal amplification for the detection of exosomal miRNA extracted from human blood [138]. The stable SERS probes of Au@R6G@AgAu nanoparticles were prepared by attaching R6G on GNPs and then encapsulated in AgAu alloy shell nanoparticles. The sensitivity of this sensing system was notably improved by the combination of stable SERS intensity and signal amplification. Similarly, Kwizera *et al*. fabricated a SERS-based sensing platform for quantitative determination of exosomal miRNAs on the basis of a uniform plasmonic head-flocked gold nanopillar substrate, which could generate multiple hotspots after hybridization between short oligonucleotides (Fig. 5C) [139]. SERS tags are essential and indispensable to SERS biosensors. Thus, they have to be elaborately designed to improve sensitivity. In addition, portable Raman spectrometer is highly needed to make biosensors become commercialized for exosome detection in point-of-care diagnosis.

Microfluidic-based biosensors

Serving as novel manipulation techniques, microfluidic-based technologies have exhibited great promise in biological applications over the last several decades by manipulating fluids at submillimeter scale [140]. Microfluidic devices have the advantage of high-throughput analysis of biomolecules with high sensitivity, low reagent consumption, and short analysis time. With the rapid development of microfluidic techniques, excellent microfluidic-based biosensors integrated with various signal readout techniques have been developed [141]. For example, Kanwar *et al*. proposed an integrated microfluidic platform fabricated in PDMS. The platform was named ExoChip, which was modified with a specific anti-CD63 antibody to capture exosomes, followed by staining the captured exosomes with a fluorescent carbocyanine dye (DiO) and quantitation of the concentrations by using a standard plate-reader [142]. This ExoChip allowed simultaneous capturing and quantification of circulating exosomes directly from blood serum. A microfluidic exosome analysis platform based on a new GO/PDA nano-interface was designed and fabricated by Zhang *et al*. to achieve ultrasensitive analysis of cancer cell-derived exosomes (Fig. 6A) [143]. In this microfluidic chip, the GO-induced formation of a 3D nanoporous PDA surface was prepared by the microfluidic layer-by-layer deposition of GO and PDA, which greatly improved the efficiency of exosome immuno-capture and effectively suppressed non-specific adsorption. On the basis of this nano-interface, an ultrasensitive assay was achieved for exosome detection, with a very low detection limit of 50 particles/μL.

The combination of colorimetric signal output and microfluidic technology may allow the visualization of exosomes with excellent performance. Vaidyanathan *et al*. proposed multiplexed microfluidic device by using a tunable alternating current electrohydrodynamic methodology for highly specific capture and detection of multiple exosomes (Fig. 6B) [144]. In this method, exosomes were captured on the surface of functionalized electrodes, and absorbance measurement of the colorimetric solution was performed for quantification. Woo *et al*. presented a rapid, label-free, and highly sensitive Exodisc platform that could be used to detect bladder cancer-related exosomes in a microfluidic chip with two-stage filtration process [145]. The signal of the microfluidic device could be recorded using a smart phone camera for colorimetric detection of the exosomes captured by the filter membrane to make the detection system convenient (Fig. 6C) [146]. One advantage of such a "lab-on-a-chip" standardization sequence is that the automation of separation, labeling, and detection operations could be performed on an effective platform.

SPR-based microfluidic techniques combine microfluidics and SPR, with advantages of rapid detection, portability, and label-free detection. Zhu *et al*. designed a SPR-based microfluidic chip for real-time and label-free quantification of multiple exosomal proteins without enrichment or purification process [147]. With the capture of exosomes by antibody microarrays modified on the gold surface, the refractive index of this biosensor could generate a huge change, which could be detected via SPR imaging. Meanwhile, nuclear magnetic resonance (NMR) is an important technique used in chemical and biomedicine analysis. The combination of NMR and microfluidics for exosome detection has also been developed with high reproducibility and sensitivity [148]. Besides, some electrochemical microfluidic-based biosensors that integrated electrochemical techniques with microfluidic platforms have shown remarkable performances in the detection of exosomes, with high sensitivity [149]. Microfluidic-implemented biosensors have shown better performances in terms of shorter operating time, smaller sample consumptions, and higher automation than bulk systems.

Conclusion and perspectives

Early diagnosis of cancer is important and indispensable in the improvement of therapeutic efficacy and patient survival. Selecting appropriate and specific biomarkers with sensitive and selective assay methods is essential. Exosomes containing various contents play important roles

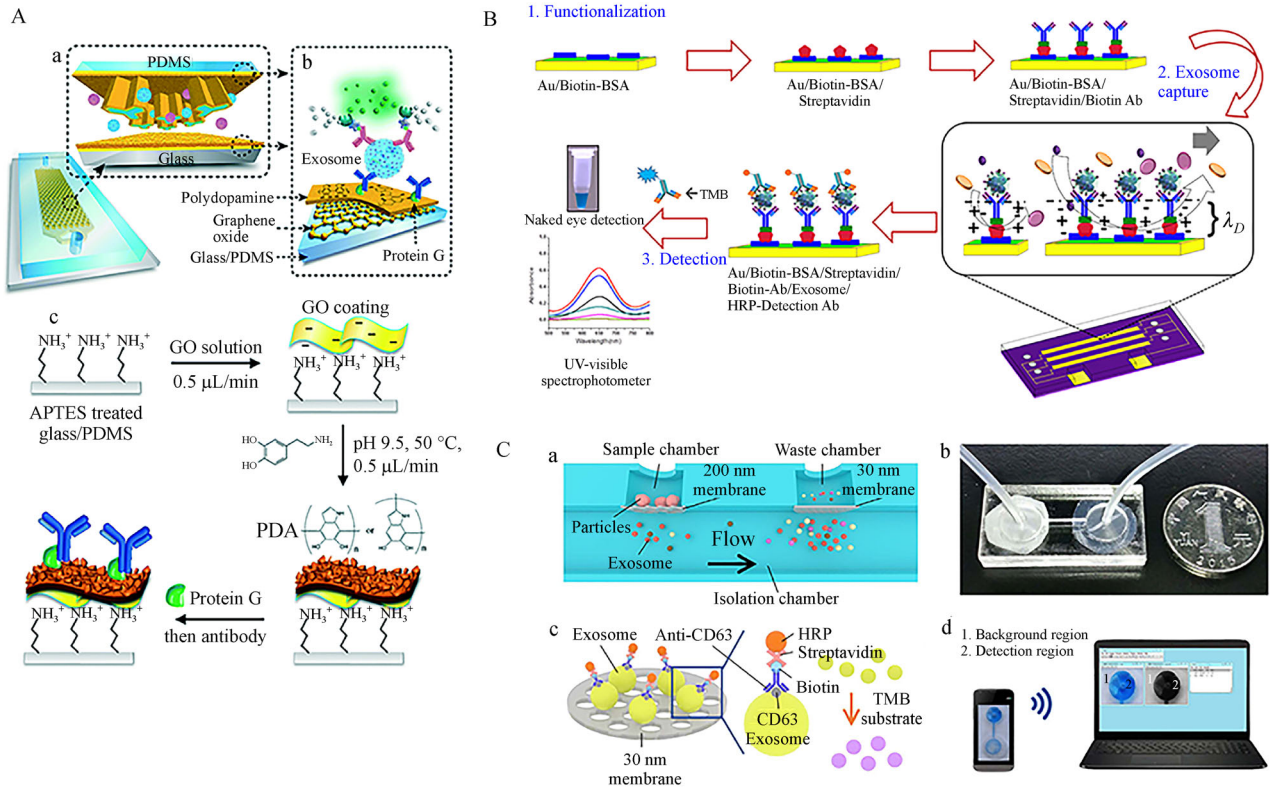

Fig. 6 Schematic of representative microfluidic-based biosensors. (A) Detection of circulating exosomes by using a nanostructured GO/polydopamine functionalized microfluidic platform. Description of (a) a single-channel PDMS/glass chip containing an array of Y-shaped microposts and (b) channel and microposts coated with GO and polydopamine (PDA) as a nanostructured interface. (c) Procedure for surface functionalization of microfluidic chips. (Reproduced with permission from Ref. [143]. Copyright 2016 the Royal Society of Chemistry.) (B) Colorimetric detection of exosomes by using a tunable alternating current electrohydrodynamic methodology. (Reproduced with permission from Ref. [144]. Copyright 2014 American Chemical Society.) (C) Detection of exosomes from urine by using an integrated double-filtration microfluidic device. (a) Diagram of a double-filtration microfluidic device for isolation and detection of exosomes. (b) Size description of an assembled double-filtration device. (c) Diagram of direct ELISA for on-chip exosome detection. (d) The ELISA result is imaged via a smart phone, and then data analysis is conducted on a laptop. (Reproduced with permission from Ref. [146]. Copyright 2017 Nature Publishing Group.)

in the development of cancer, hence considered as promising biomarkers. Significant progress has been made in the design and fabrication of various types of biosensors for the detection of exosomes, providing the basis for early and widespread screening of cancer. Herein, different types of biosensors for detecting cancer-derived exosomes in recent years were reviewed, with an emphasis on the sensitivity and selectivity of biosensors and their clinical application. Biosensor-based methods for exosome assays are notably becoming a promising alternative in the early diagnosis because of advantages, such as condensed detection time, simple operation, and low cost. However, some challenges are still needed to be addressed to convert laboratory methods to clinical applications.

For the future development of biosensors in detecting exosomes, researchers need to focus on the sensitivity, selectivity, biocompatibility, and anti-interference in clinical testing, because samples always contain a large number of cells, nucleic acids, proteins, and other components. Thus far, some multi-signal amplification methods have been proposed to improve sensitivity. Besides, the physiology and pathology are complex in cancer cells. Thus, the selectivity of diverse exosome markers for targeting different cancer types should be given attention. Combination of molecular pathological epidemiology, genomics, proteomics, and big data science could help acquire an enhanced understanding of the etiologies and pathogenesis of cancer and then select appropriate exosome markers [37,150]. Moreover, the biocompatibility of the sensing layer is critical to avoid immune response, especially in long-term detection *in vivo*. The future of biosensors for disease detection is directly oriented to a larger patient population. Therefore, devices need to be portable, user friendly, and inexpensive. Early screening of cancer could be in a new era with the rapid progress of biosensor-based assay technology, which

could considerably improve the therapeutic effects and patient survival.

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Compliance with ethics guidelines

Ying Deng, Zhaowei Sun, Lei Wang, Minghui Wang, Jie Yang, and Genxi Li declare that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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