Aptamers as Recognition Elements for Electrochemical Detection of Exosomes

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Exosome analysis is emerging as an attractive noninvasive approach for disease diagnosis and treatment monitoring in the field of liquid biopsy. Aptamer is considered as a promising molecular probe for exosomes detection because of the high binding affinity, remarkable specificity, and low cost. Recently, many approaches have been developed to further improve the performance of electrochemical aptamer based(E-AB) sensors with a lower limit of detection. In this review, we focus on the development of using aptamer as a specific recognition element for exosomes detection in electrochemical sensors. We first introduce recent advances in evolving aptamers against exosomes. Then, we review methods of immobilization aptamers on electrode surfaces, followed by a summary of the main strategies of signal amplification. Finally, we present the insights of the challenges and future directions of E-AB sensors for exosomes analysis.

Keywords Aptamer; Exosome; Biosensor; Diagnosis; Biomolecular recognition

1 Introduction

Exosomes are nanoscale membrane-bound lipid vesicles with a diameter ranging from 30 nm to 200 nm, which shuttle diverse biologically active substances from parental cells to other cells[1]. They can be found in various bodily fluids, such as blood, urine, saliva, cerebrospinal fluid, and breast milk with high abundance and stability[2]. Specifically, the composition of exosomes including lipids, proteins, mRNA/miRNA and DNA fragments reflects most closely the physiological and pathological status of originated cells[3,4]. Because of these attributes, exosomes are considered ideal noninvasive biomarkers for diagnosing various diseases(*e.g.*, pregnancy, cardiovascular diseases, central nervous system-related diseases, and cancers), and monitoring the patient responses during therapy[3,5]. Especially, recent studies have shown that quantitative detection of exosomes and their cargoes is important for identifying physiological and pathological markers[6−8]. Traditional qualitative techniques, such as Western blotting, enzyme-linked immunosorbent assays, and mass spectrometry typically require a large sample volume, extensive processing, and specialized instrumentation. Besides, it is still a challenge to detect the low abundance of biomarkers in exosomes with these methods[9,10]. To overcome these drawbacks, new generations of biosensors are under development in the field of exosomes analysis, such as fluorescence integrated microfluidics biosensors[11], surface-enhanced Raman scattering(SERS) biosensors[12], electrochemical biosensors[13,14], and field-effect transistor(FET) biosensors[15]. Among those techniques, electrochemical biosensors have great potential for exosomal proteins analysis by virtue of high selectivity, robustness, and ease of use.

Most electrochemical biosensors utilize antibodies or aptamers to achieve rapid, sensitive, and selective detection of exosomes *via* specific molecular recognition. The property of targets is determined by measuring the changes in electric potential or current[16−18]. Concerning antibodies, aptamers, smart and promising molecular recognition ligands, own many advantages for diagnostic applications, including stronger binding affinity to targets, higher stability against temperature and pH changes, and low cost[5,19−21]. Furthermore, aptamers can be chemically synthesized in a cell-free system, and easily be labeled or modified according to the specified requirement[22,23]. Based on these features, electrochemical aptamer-based(E-AB) sensors have received significant attention in exosome profiling recently.

Recently, various E-AB sensors for detecting exosomes have been successfully used in clinical applications. However, such devices have been hobbled for the limited variety of aptamers, complicated immobilization process, and insufficient sensitivity. This review will focus on the recent progress on aptamer based electrochemical analysis of exosomes over the past five years. We first introduce developments of evolving aptamers against exosomes. Then, we summarize methods for immobilization aptamers on electrode surfaces and review the main strategies of signal amplification. Finally, we also provide the insights of the challenges and future directions of E-AB sensors for exosomes

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analysis.

2 Development of Aptamer Selection Technology for Exosomes

Aptamers are synthetic single-stranded DNAs or RNAs with a length of about 25—80 bases that form unique secondary and tertiary structures upon binding to targets. They are selected from an *in vitro* process known as systematic evolution of ligands by the exponential enrichment(SELEX) technology[24]. Several modified SELEX methods, such as counter SELEX, capillary electrophoresis SELEX, microfluidic SELEX, cell SELEX, *in vivo* SELEX and high-throughput sequencing SELEX have been developed to shorten selection time and enhance hit rate[25—28]. Typically, SELEX process of selecting an aptamer for a particular molecular target consists of four steps: pool generation, selection, amplification, and isolation. The target types are ranging from metal ions to small molecules, proteins, and complex target mixtures(*e.g.*, viruses, bacteria, whole cells, and animals).

In recent years, numerous aptamers have been selected and utilized as novel biological recognition molecules toward exosome associated membrane proteins. As shown in Fig.1, among those exosome associated membrane proteins, tetraspanin proteins(*e.g.*, CD9, CD63, and CD81), a superfamily of proteins with four transmembrane domains, which are highly expressed in exosomes, and thus, are suitable for the targets of aptamer binding[29]. However, tetraspanins are not uniquely expressed in exosomes alone[30]. Beyond tetraspanins, membrane transport and fusion proteins(Annexins, flotillin, Rab GTPase family), proteins involved in multivesicular body biogenesis(*e.g.*, Alix, TSG101), and lipid-bound extracellular proteins(*e.g.*, lactadherin), are also adapted for the targets of aptamer binding. And these proteins are also not homogeneous across exosomes from different sources. In addition, the specific transmembrane
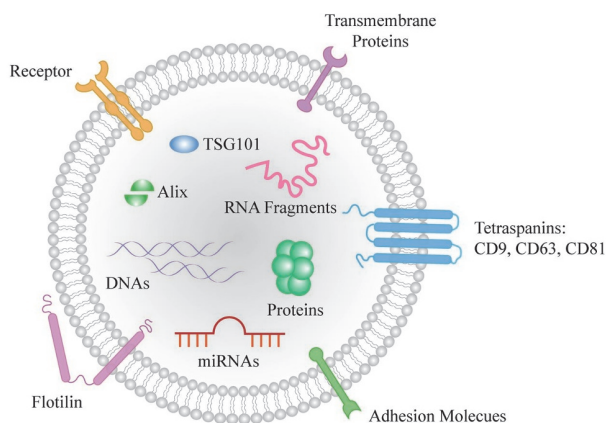

Fig.1 Structure and contents of exosome
Typically, the lipid bilayer membrane structures of exosomes carry amount of functional proteins.

protein receptors and adhesion proteins(*e.g.*, HER2, EGFR, EpCAM) are associated with the normal physiology and pathogenesis of many diseases, which are used as important pathophysiological exosome biomarkers. Therefore, aptamers against those proteins are mostly used in the application of disease diagnosis and treatment monitoring.

The aforementioned aptamers are mostly selected by iterative incubation with known pure exosome-surface marker proteins, leading to the scope for their application limited[31]. Condorelli *et al.*[32] recently developed a novel differential SELEX methodology, which is named Exo-SELEX. Similarly to CELL-SELEX，in the Exo-SELEX, no prior knowledge of exosome-surface marker proteins is required. At each round of the selection, exosomes derived from either primary normal or cancer breast epithelial cells are used as complex targets for negative and positive selection, respectively. During the selection process, exosomes were conjugated with magnetic beads for incubation with RNA library, and the binding ability of the selected aptamer was characterized by a direct-enzyme-linked oligonucleotides assay. This selection strategy will be widely applied in targeting to cancer cells-derived exosomes. However, the selected aptamer against protein was not determined in this work.

In contrast with tumor-derived exosomes, other diseases related exosomes are mainly isolated from human biofluids or tissues, which is more technically challenging. In our previous work, we[33] used a brain slice-based SELEX method to enrich and select specific aptamers in a mouse model of ischemia. In this work, we separately used frozen ischemia brain slices and sham brain slices as targets for positive and negative selection. The fluorescence intensity of ssDNA in tissue slice was monitored by confocal imaging to evaluate the enrichment process of the selection. Finally, we selected a aptamer that could specifically bind to schemic brain slice, and identified that the target protein of the selected aptamer was previously detected in extracellular vesicles. So this method has the potential to be used for selecting aptamers targeting to exosomes. Based on this, exosomes isolated from tissue slices of model animals could be further applied as targets for aptamer selection.

Recently, to increase the nuclease resistance and binding affinities of aptamers, modified nucleotide bases, sugar rings, or phosphates have been produced in the aptamers[24]. For example, Click-SELEX, which was developed by Pfeiffer *et al.*[34], combined click chemistry with nucleic acid libraries and *in vitro* selection procedures. In the Click-SELEX, the modified DNA is compatible with the conventional steps of the selection procedure.

3 Aptamer Immobilization Strategies

In E-AB sensors, aptamer is used as the biorecognition element

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on electrode surface for converting the recognition of a biomolecule into electrical signals. The recognition and binding efficiency of the aptamer toward biomarkers can be profoundly affected by the fabrication step of immobilization. Now, various direct immobilization methods and free immobilization methods have been developed to improve the detection sensitivity.

3.1 Direct Immobilization Strategies

In conventional fabrication method, aptamers are directly immobilized on the electrode surface by the utilization of electrostatic interaction, covalent binding or streptavidin/biotin interaction. Recently, DNA nanostructures, poly adenine and the targets have been used to assist the aptamer immobilization.

The negatively charged hydrophilic sugar-phosphate backbone makes DNA probe directly immobilize on the positively charged electrode surface by electrostatic interaction, which have been widely applied in electrochemical DNA hybridization sensors in the past[35]. However, in this method, the DNA molecules are attached to electrode surface through multiple phosphate sites, resulting in poor recognition efficiency[36]. Now, using one-point attachment is the more considered aptamer immobilization strategy. Originally, E-AB sensors are mainly fabricated by using gold electrode surfaces for their advantages of unique physical and chemical properties. Thiolated aptamers can be efficiently immobilized on the gold electrode surface, forming a high-density monomolecular layer without further complicated modifications[37]. However, aptamers immobilized on the electrode surface should be sufficiently spaced to bind targets and subsequently fold for signal transduction. To improve the accessibility of the aptamer to the analyte, most of the aptamers are immobilized *via* thiolated short linkers[37]. Currently, mercaptohexanol(MCH) is subsequently added to displace the nonspecifically adsorbed parts of the aptamers and guarantees their vertical orientation[38,39]. By this method, Zhou *et al.*[40] immobilized CD63 aptamer on the gold electrode surfaces, and integrated them into a microfluidic system for capturing exosomes. The limit of detection(LOD) is $1×10^3$ particles/μL, which decreased 100-fold compared to those of commercial immunoassays relying on anti-CD63 antibodies. Otherwise, covalent surface modifications of graphene-based materials are also easily realized. The aptamers can be immobilized on the electrode surface *via* simple click chemistry or cycloaddition reaction[41,42].

Recently, Tan's group[43] successfully used a DNA nanostructure to regulate the aptamer orientation and spatial arrangement for improving biomolecular recognition efficiency[Fig.2(A)]. Compared with traditional aptamer-based biosensors, the nanotetrahedron-assisted aptasensor could detect exosomes with 100-fold higher sensitivity. Furthermore,

there are some new methods that have been developed to make the aptamer more conveniently immobilized on the gold surface. For example, Fan *et al.*[44] demonstrated that poly adenine(polyA) can not only strongly bind to AuNP surface but also effectively block nonspecific DNA-Au binding[Fig.2(B)]. Without any modification, this method could be helpful for precisely controlling the orientation and density of aptamers on gold electrode surface. Liu *et al.*[45] described a generalizable method of using target-assisted aptamer immobilization, which has greatly improved the sensitivity and signal-to-noise ratio of E-AB sensors.

3.2 Free Immobilization Strategies

Directly immobilizing aptamers on the electrode surfaces is time-consuming, expensive, and labor-intensive. The recognition and binding ability of the aptamer toward exosome are limited on the solution-electrode interface[38,46]. To circumvent the immobilization processes, free immobilization electrochemical sensing platforms have been developed with a higher detection sensitivity. Among these methods, sandwich assay based on "capture probe-exosome-aptamer" format is mostly employed to improve the detection sensitivity. For example, Huang *et al.*[47] first immobilized CD63 antibodies onto the electrode to capture all exosomes, and then the aptamers against the MUC1 protein in free solution were added to specifically identify and quantify gastric cancer exosomes. Xu *et al.*[48] also developed a free immobilization sandwich assay of "MB/Tim4-exosomes-CD63 aptamer" type. In that sensing platform, tumor-derived CD63-positive exosomes were enriched by Tim4-modified magnetic beads and further analyzed by CD63 aptamer with an LOD of *ca.* 4 particles/μL[Fig.2(C)]. Li's group[49] also developed an immobilization-free and label-free electrochemical platform for ultrasensitive detection of cancer-derived exosomes with a
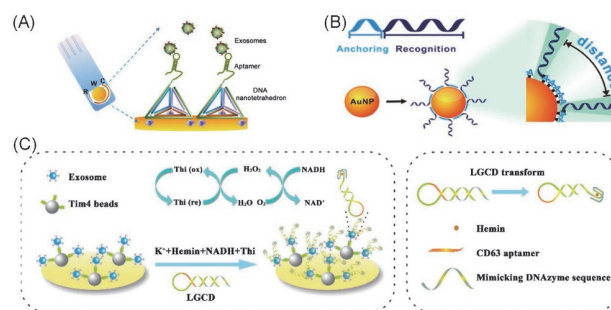

Fig.2 Schematic illustration of aptamers immobilization methods

Using DNA nanostructures(A) or poly adenine(B) to control the aptamer orientation and spatial arrangement on gold surface; (C) using target-assisted aptamer immobilization to improve sensitivity and signal-to-noise ratio.

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low LOD of 12 particles/μL, which exhibited outstanding features of simplicity, rapidness, cost-efficiency, and easy manipulation.

4 Strategies of Signal Amplification

The low concentration of diseases related exosomal proteins in body fluids has led to an urgent demand of highly sensitive detection technologies. Developing facile and effective signal amplification strategies for exosome detection is highly desirable in noninvasive early diagnosis. In electrochemical sensors, amplification strategies are mainly based on introducing nanomaterials on the electrode surface or adding signal amplification moieties in aptamers.

4.1 Nanomaterials-based Signal Amplification

Nanomaterials can not only provide larger recognition surface area but also promote the electron transfer on the electrode interface and amplify the detection signal, leading to ultrasensitive biosensing[18,50]. For these reasons, various nanomaterials, such as metal nanoparticles(MMP) , graphene oxide, and metal-organic frameworks(MOFs), have been introduced in exosomes detection biosensors. For example, Ti_3C_2 MXenes have advantages of good electrical conductivities, hydrophilic, large surfaces, and excellent catalytic performance[51]. The *in situ* formed AuNPs-MXenes-Aptamer hybrid nanoprobe was synthesized and applied in an electrogenerated chemiluminescence biosensor by Liu's group[52][Fig.3(A)]. The AuNPs-MXenes-Aptamer nanoprobe presented highly efficient recognition of exosomes and provided naked catalytic surfaces with high electrocatalytic activity of AuNPs. The detection limit of exosomes was improved to 30 particles/μL. Besides, Zhou *et al.*[53] presented a simple multiplexed electrochemical sensor as a platform for simultaneously detecting different proteins on vesicles by direct electro-oxidation of aptamer modified metal nanoparticles(MNPs). This sensor exhibited an LOD of 50 exosomes/sensor with a low sample consumption(25 μL).

Significantly, novel nanomaterials coupled with aptamer-based amplification methods could highly improve the detection sensitivity. Li's group[54] developed a simple and inexpensive paper-based E-AB sensor for ultrasensitive and quantitative determination of cancer-derived exosomes [Fig.3(B)]. In that sensor, the DNA probe containing CD63 aptamer sequence was strongly bound to Zr-MOFs *via* the formation of Zr—O—P bonds, which could recognize CD63-positive exosomes and trigger a conformational change, initiating upstream hybridization chain reaction and formation of DNAzyme. Benefiting from this amplification method, the developed paper-based exosome biosensor is
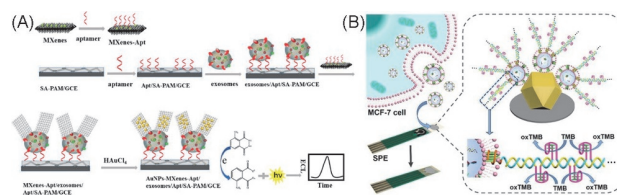

Fig.3 Schematic illustration of using nanomaterials to amplificate the electrochemical signals for exosomes detection

(A) The *in situ* formed AuNPs-MXenes-Aptamer hybrid nanoprobe was used in electrogenerated chemiluminescence sensor for exosomes detection; (B) Zr-MOFs functionalized screen-printed electrode for exosomes detection.

(A) Reprinted with permission from Ref.[52], Copyright 2020, American Chemical Society; (B) reprinted with permission from Ref.[54], Copyright 2021, American Chemical Society.

capable of detecting exosomes with a detection limit down to 5 particles/μL.

Our group[55,56] have previously fabricated several nanocomposite film modified electrodes. In these sensors, the nanofilms were not only used to improve the effective surface area and the conductivity of the electrodes, but also prevented the electrode surface passivation caused by the non-specific adsorption of proteins and other substances *in vivo*. The nanofilms may offer a new potential nanomaterials for amplifying the electrochemical signals of exosomes.

4.2 Aptamer-based Signal Amplification Strategies

Aptamers with high programmability and flexibility are easily coupled with isothermal amplification technologies, such as rolling circle amplification(RCA), hybridization chain reaction(HCR), DNA walker, etc. The aptamer-based signal amplification strategies can be divided into direct amplification type and competitive amplification type.

4.2.1 Direct Amplification Type

In the direct amplification assay, the aptamer-exosome interaction would directly trigger DNA amplification/assembly or enzyme-catalyzed reaction. These methods have advantages of rapid readout, simple manipulation, and high sensitivity, but also cause much higher background signals[57].

The concept of HCR was introduced by Dirks *et al.*[58] in 2004, in which DNA acts as an amplifying transducer for biosensing. Due to its versatility and simplicity, this isothermal enzyme-free DNA amplification technique has been an attractive tool for DNA nanotechnology, biosensing, and biomedicine research. Therefore, various E-AB sensors based on HCR amplification technique have been investigated for the detection of exosomes. At first, Ye's group[59] and Li's group[60] successfully used the cholesterol-lipid interaction to anchor

DNA probe onto exosome membranes for triggering a DNA hybridization chain reaction(HCR). Then, An *et al.*[61] developed another new efficient anchoring method for forming long self-assembled DNA concatemers on exosome surface[Fig.4(A)]. Specifically, CD63 aptamers as exosome capture probes were firstly immobilized onto the DenAu/rGO/GCE electrode. Then the azide-labeled DNA probes used for HCR amplification were conjugated to exosome membrane proteins through a copper(I)-catalyzed click chemistry reaction. In this way, the E-AB sensor for tumor exosomes detection has an LOD of 96 particles/μL.

RCA is an isothermal DNA amplification technique, which is able to generate long single stranded DNA with repetitive functional sequences(*e.g.*, DNAzymes, restriction enzyme sites, and functional moieties) for signal amplification. As RCA reaction can achieve a more than billion-fold amplification within 1—2 h at room temperature, this technology has been widely employed in an E-AB sensor for signal amplification. For example, Huang *et al.*[47] fabricated a label free aptasensor based on RCA reaction to detect gastric cancer exosomes[Fig.4(B)]. In this case, various exosomes were captured by anti-CD63 antibodies in a gold electrode. The DNA probe containing an MUC-1 recognition aptamer and an RCA primer could bind to the gastric cancer exosomes and initia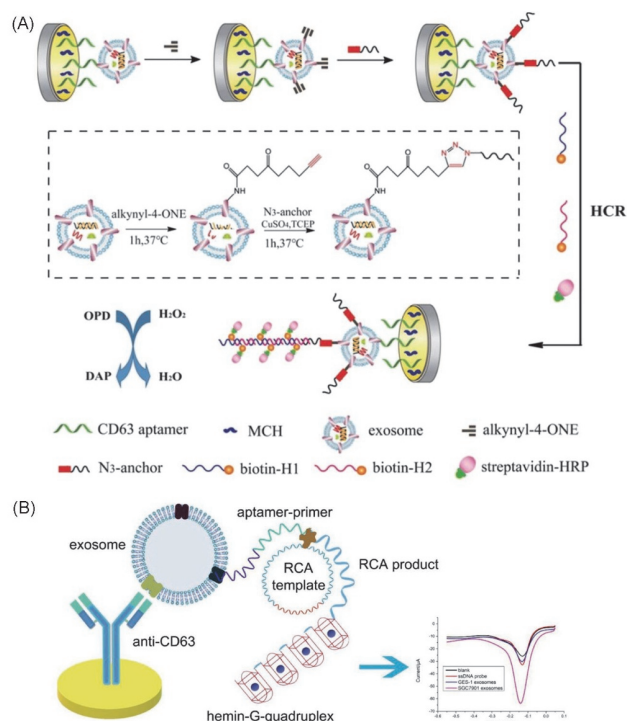te the RCA reaction, producing multiple G-quadruplex units to catalyze amplification of an electrochemical reaction. This aptasensor exhibited a detection limit of *ca.* 1 particles/μL and a linear response range from 4.8 particles/μL to 4.8×10^3 particles/μL.

4.2.2 Competitive Amplification Type

In a competitive amplification assay, the recognition of aptamer with exosome would release multi-DNA strands, which converts exosome capture to ssDNA detection. In contrast to the direct amplification method, the competitive method shows the advantages of low background signals, and is versatile to muti-exosomes detection by changing the aptamer probes. For example, Dong *et al.*[62] reported an amplification strategy for exosome detection based on aptamer recognition induced multi-DNA release and cyclic enzymatic amplification[Fig.5(A)]. In this project, exosomes were captured by prostate specific membrane antigen(PSMA) aptamers modified on magnetic bead. Then three kinds of messenger DNAs(mDNAs) hybridized with the aptamers were released to amplify the signal, leading to a detection limit of 70 particles/μL.

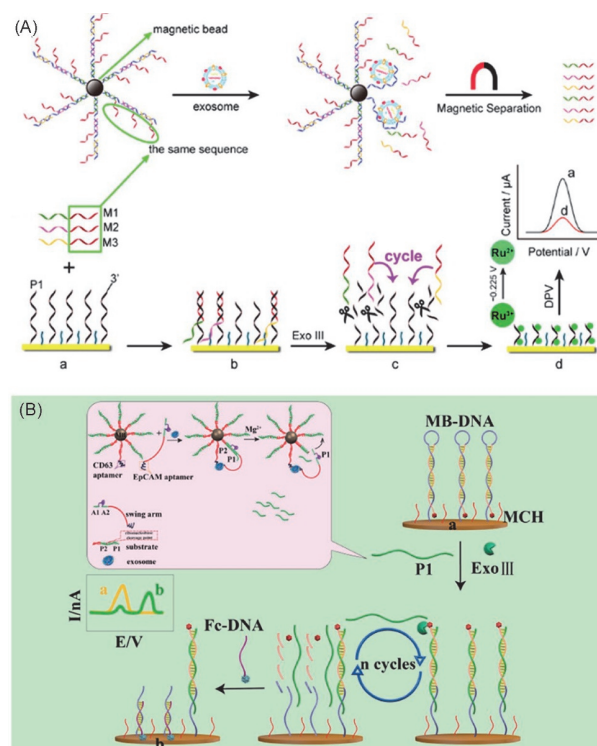DNA walker, as another new attractive competitive amplification strategy, has been widely combined into an

Fig.4 Schematic representation of aptamer-based direct signal amplification in E-AB sensor for exosomes detection

(A) Combining click chemistry and HCR signal amplification for exosomes detection; (B) using the RCA reaction to produce multiple G-quadruplex units for catalyzed amplification.

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Fig.5 Schematic representation of aptamer-based competitive signal amplification in E-AB sensor for exosomes detection

(A) Exosomes detection based on aptamer recognition induced multi-DNA release and cyclic enzymatic amplification; (B) ratiometric electrochemical detection of exosomes by combing amplification of 3D DNA walker and Exo III-assisted strands recycling.

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aptasensor for sensitive determination of exosomes. Typically, after aptamer and exosome interaction triggered the motion of DNA walker, and DNA walker moved independently along the designed oligonucleotide tracks driven by DNAzyme or strand displacement reaction(SDR), which released a large quantity of single-stranded DNAs for signal amplification. For example, Zhao et al.[63] developed an ultrasensitive approach for ratiometric electrochemical detection of exosomes by combing amplification of 3D DNA walker and Exo III-assisted strands recycling[Fig.5(B)]. The movement of the DNAzyme along the 3D tracks was triggered through the recognition of two DNA aptamers with exosomes. Under the optimal conditions, the detection limit of 13 particles/μL was obtained with excellent selectivity. In addition, the DNA walker owned more "legs" has a larger walking area, a faster walking kinetics and a higher amplification efficiency, which have been demonstrated in DNA[64] and MiroRNA detection assays[65].

In addition, clustered regularly interspaced short palindromic repeat(CRISPR), a popular gene editing system, provides a new approach for signal amplification[66]. The strategy of integrating CRISPR/Cas12a system with aptasensors has been proposed to improve analytical specificity and sensitivity[67]. For instance, Zhao et al.[68] developed a CRISPR/Cas12a-based fluorescent aptasensor for detecting CD63-bearing exosomes. Xing et al.[69] combined HCR amplification in the CRISPR/Cas12a-based fluorescent aptasensors for direct high-sensitivity detection of tumor-derived extracellular vesicle proteins. Han et al.[70] developed a CRISPR/Cas12a-based E-AB sensor for ultrasensitive detection of a COVID-19 nucleocapsid protein, which benefited from the high-efficiency *trans*-cleavage activity of Cas12a and sensitive performance of E-AB sensors. Although CRISPR/Cas12a-based E-AB sensors for exosomes detection have not been reported now, it is reasonable to believe that this strategy will be popular in the near future.

5 Summary and Outlook

Due to the high selectivity, robustness, and ease of use, E-AB sensors for exosomes analysis are emerging as a promising class of analytical devices for diagnosis and therapy guidance of diseases. In summary, the developments of E-AB sensors for detecting exosomes were focused on aptamer selecting approaches, aptamer immobilization methods, and strategies of signal amplification. Despite the advantages and the clinic application potential, there are still several challenges and critical technological problems for researchers to solve in the future.

First of all, exosomes are not only served as a predictor for cancer diagnosis, but also suitable for other diseases diagnosis, such as diabetes, cardiovascular disease, neurodegenerative disorders and pathogen infection[71,72]. Detection of those diseases related exosomes is an actual analytical challenge for their low abundance. To satisfy this clinical demand, it is necessary to select aptamers targeting to other types of exosomes and fabricate the corresponding E-AB sensors.

Then, multi-aptamers based sensors have offered high sensitivity and specificity to analyze exosomes[73]. Combing with advanced DNA computation technology will provide more detailed information on exosomes, and enable abundant possibilities for the development of clinically applicable E-AB sensors. More aptamers are necessarily required to be selected in the future.

At last, we believe future work will focus on expanding convenient E-AB sensors for point-of-care(POC) diagnostics, which have attracted increasing attention in the area of global health. Recently, Song's group[74] has used a smartphone integrated with electrochemical biosensor for the quantification of exosomes. More novel hand-held type devices for exosome detection will be developed in the near future.

Conflicts of Interest

The authors declare no conflicts of interest.

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