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Research advances for exosomal miRNAs detection in biosensing: From the massive study to the individual study

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A B S T R A C T

Exosomal miRNAs (exo-miRNAs) derived from cancer cell play a vital role in cancer development and can be considered as a potential biomarker for cancer diagnostic. However, the shortage of sensitive preparatory and analytical technologies limits their clinical applications. Thus, sensitive and reliable sensing of exo-miRNAs is essential in medical diagnostics. Mounting number of biosensor strategies are thus actively being proposed to deal with these challenges, including nanoparticle, isothermal amplification, and microfluidic chips. Moreover, some delivery strategies have been found for individual exo-miRNAs detection, such as membrane fusion, nano-sized probe and membrane denatured. Therefore, we summarize and discuss the recent progress of emerging biosensor assays for sensing exo-miRNAs. In addition, challenges and outlook for exo-miRNAs detection are considered.

1. Introduction

MiRNAs are a family of small (22–24 nucleotides), single-stranded, non-coding RNA molecules (Ambros 2004; Cortez et al., 2011; Jeffrey 2008). These highly conserved functional RNAs bind to target mRNAs for modulating gene expression, resulting in target transcripts degradation (Correia de Sousa et al., 2019). Such regulatory mechanisms enable miRNAs to mediate many regulatory pathways, including organogenesis (Hayashi and Hoffman 2017), cell proliferation (Sohn and Park 2018)and apoptosis (Stobiecka et al., 2019; Zhang et al., 2019), fat metabolism (Aryal et al., 2017), and viral replication (Trobaugh and Klimstra 2017).

Exosomes are heterogeneous, lipid bilayer-membrane particles with a size range from 50 to 150 nm (Laulagnier et al., 2004; Melo et al., 2015; Valadi et al., 2007). They contain proteins, mRNAs and miRNAs, are secreted by almost all kinds of cells, especially by cancer cells (Hannafon et al., 2016; Joyce et al., 2016). Originally thought to be "cellular dust", they are now proven as an important medium for intracellular communication, information exchange and disease development (Tomasetti et al., 2017; Wortzel et al., 2019). Recent studies have demonstrated that valuable miRNAs are usually concentrated in exosomes (Sun et al., 2018; Zhang et al., 2015). Compared with the circulating miRNAs, exo-miRNAs are relatively stable due to the protection of lipid bilayer-membrane (Hessvik and Llorente 2018). Abnormal expression miRNAs can be found in exosomes secreted by various cancer cells, such as breast cancer, stomach cancer, and esophageal cancer (Li et al., 2019; Ratajczak and Stobiecka 2020; Santos et al., 2018; Zhang et al., 2017). Thus, exo-miRNAs appear to be a promising biomarker in early cancer diagnosis and prognostic processes.

At present, various methods for exo-miRNAs detection have been reported (Drula et al., 2020; Lee et al., 2018; Ramshani et al., 2019). The three most widely used assays include reverse transcription-polymerase chain reaction (RT-PCR) (Manier et al., 2017), digital PCR (Wang et al., 2019), and next-generation sequencing technology (NGS) (Selmaj et al., 2017). Although these methods have excellent analytical performance, they are still suffering some defects for clinical applications. RT-PCR can be used for detecting exo-miRNAs, but it has a relatively low sensitivity and expensive equipment (Zhou et al., 2018). Digital PCR can detect

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trace amounts of miRNAs, but high cost and complex operation is needed (Armstrong et al., 2015). NGS usually require tedious pretreatment and multistep reactions (Stevanato et al., 2016). Compared with these methods, biosensor is sensitive and rapid, which need no complicated operation and expensive instrument (Luo et al., 2020; Pang et al., 2019). Moreover, biosensors can be used for detecting exo-miRNAs in single exosome (Chen et al., 2018; Liu et al., 2020a). At present, only two reviews describe exo-miRNA detection techniques (Drula et al., 2020; Shao et al., 2018). In Shao's review, one page was used for introduction the exo-miRNA detection. In Drula's review, only ddPCR and Surface-Enhanced Raman Scattering (SERS) were referenced for exo-miRNA detection, which is far from summarizing this field. Thus, we review research advances for exo-miRNAs detection in biosensing, ranging from the group to the individual. This review particularly focuses on describing new biosensor strategy and platform advances for exo-miRNAs detection.

2. Detection assays by exo-miRNA extraction

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is "gold standard" for exo-miRNAs detection (Wu et al., 2018). It usually requires RNA extraction, reverse transcription to synthesize cDNA, and PCR-amplification. The whole process needs rigorous temperature management and specialized instruments (Hu et al., 2018; Yang et al., 2018). Herein, scientists have established many strategies, including nanomaterials (Boriachek et al., 2018; Ma et al., 2018), isothermal amplification (Joshi et al., 2015; Liu et al., 2020b; Tang et al., 2020; Zhao et al., 2015) and microfluidic (Cheung et al., 2018; Goda et al., 2012; Richards et al., 2017) for exo-miRNAs detection.

2.1. Nanomaterials

Nanomaterials are a class of material with nanoscale size, including metal, inorganic and organic nanomaterials. It holds great potential in various fields because of its inherent advantages: large surface, good conductivity and small size (Lee et al., 2019). Furthermore, the biological properties of nanomaterials greatly increase its value in biosensing, such as fluorescent characteristic, enzyme-mimic activity, and remarkable biocompatibility (Li et al., 2015). Due to advantages mentioned above, nanomaterials are introduced as carrier, optical emitter, electronic conductor and catalyzer to achieve ultrasensitive detection of exo-miRNAs.

Au nanoparticles (NPs), as a representative nanomaterial, have good performance of nucleic acid electrostatic adsorption (Masud et al., 2020). Target complementary probe as capture probe is designed with thiolated end for attachment to AuNPs via Au–S bond. The DNA/Au nanoparticles conjugate named as spherical nucleic acids (SNA) provide novel functional properties and develop a wide range of application in clinical diagnostic (Gao et al., 2020). Liu et al. proposed an electrochemical approach based on the formation of "sandwich" structure by SNA and peptide nucleic acid (PNA) (Liu et al., 2019). The complementary probes assemble onto the surface of AuNPs in dense arrangement could partially bind with target miRNAs (Fig. 1A). Target exo-miRNAs recognized by traditional SNA nanoprobe would carry large amount of negative charges, thus being hard to access DNA probe-immobilized interface because of repulsive force. To solve this problem, PNA with neutral charged peptide skeleton act as capture probe, which give rise to less charged and boost the binding efficiency with SNA-miRNA on the electrode surface. Hexaammineruthenium (III) chloride (RuHex) as electroactive substances can bind to

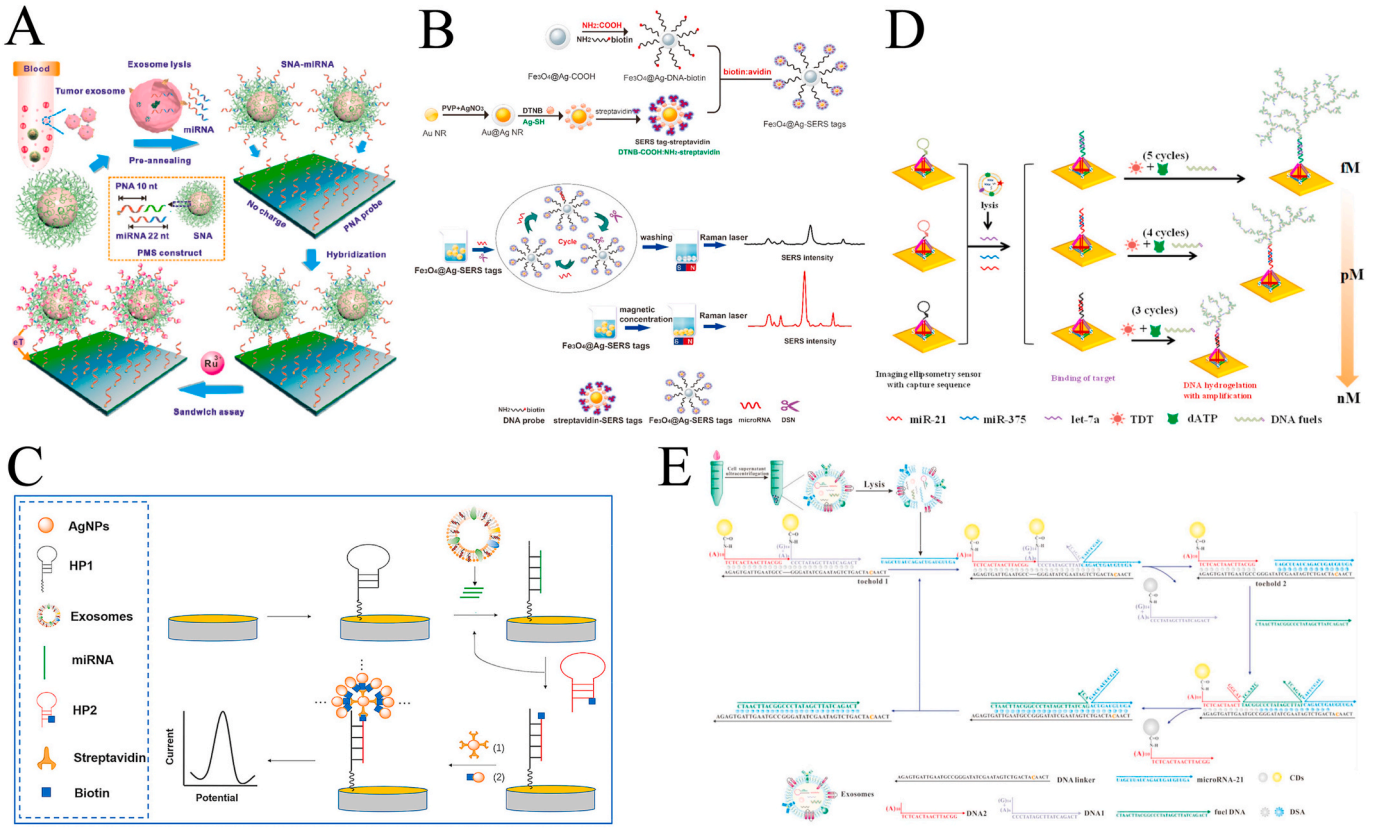

Fig. 1. (A) Electrochemical approach based on the formation of "sandwich" structure by SNA and peptide nucleic acid (PNA) (Liu et al., 2019). (B) Dual-SERS biosensor utilizing Fe_3O_4@Ag-DNA-Au@Ag@DTNB (SERS tag) (Pang et al., 2019). (C) Double-amplification strategy combining strand displacement reaction (SDR) with AgNPs (Cheng et al., 2020). (D) DNA-hydrogel for multiplexed and dynamic exo-miRNAs detection (Zou et al., 2020). (E) Radiometric fluorescence based on carbon dots (CDs) (Xia et al., 2018).

PNA-miRNA-SNA "sandwich" structures which are aggregated on the electrode surface through electrostatic adsorption. Chronocoulometric (CC) was employed to detect RuHex charges, reflecting the amounts of exo-miRNAs. The combination of SNA and PNA strategy displays excellent sensitivity and efficiency for exo-miRNAs detection. This method achieves detection limit of 49 aM with a large measuring range from 100 aM to 1 nM.

AuNPs are also used as signal reporter. To obtain powerful surface enhanced Raman scattering (SERS) signal molecules, rhodamine 6G (R6G) is conjugated with AuNPs, and coated in AgAu alloy shell. Such AuNPs composite can provide significant SERS signal for exo-miRNAs detection because of the formation of hollow interior. More recently, Pang et al. designed a dual-SERS biosensor utilizing Fe_3O_4@Ag-DNA-Au@Ag@DTNB (SERS tag) (Pang et al., 2019). In this work, Fe_3O_4 core encapsulated in Ag shell is used as recognition element and separating substrate (Fig. 1B). AuAg alloy modified with Raman reporter DTNB is used as signal molecule. Two ends of biotinylated DNA probes cross-linked with Fe_3O_4@Ag and Au@Ag@DTNB through direct absorption and biotin-streptavidin interaction respectively. In the presence of target exo-miRNAs, the DNA probes of miRNA-DNA duplexes were specifically cleaved by duplex-specific nuclease (DSN). Therefore, Au@Ag@DTNB was separated from Fe_3O_4 @Ag, the Raman signal quenched along the concentration of target increased. This dual-SERS method can be completed in one step, which means there is no need for multiple hybridization and separation.

Besides, diverse nanomaterials emerged as alternative options for testing exo-miRNAs. Cheng et al. reported a double-amplification strategy combining strand displacement reaction (SDR) with AgNPs as signal molecules, achieving sensitive detection for exo-miRNAs (Cheng et al., 2020). In this work, target miRNAs unfolded the hairpin capture probe through SDR, initiating deposition of AgNPs onto electrode surface via biotin-streptavidin interaction (Fig. 1C). Thus, the changes of electrode surface will dramatically amplify the electrochemical signal. In addition to metal-based nanomaterials, there are other types of nanomaterials also have broad prospects in sensor applications, such as nucleic acid and acridone derivative. Zou et al. adopted DNA tetrahedron for multiplexed and dynamic exo-miRNAs detection (Zou et al., 2020). DNA tetrahedron with capture probe were immobilized onto Au-film, as target exo-miRNAs exists, the hairpin structure of capture probe would unlock and expose extension site, where terminal deoxynucleotidyl transferase (TdT) specifically work (Fig. 1D). After several cycles, DNA-hydrogel was generated on the Au film. Then, the target exo-miRNAs could be quantificated via monitoring the thickness change of a thin film with imaging ellipsometry. Besides DNA hydrogel, quantum dot is another nano-material which can significantly improve the output of signal. Such as, Xia et al. constructed radiometric fluorescence based on carbon dots (CDs) (Xia et al., 2018). Based on the superior properties of CDs, the concentration of exo-miRNAs could be detected as low as 3.0 fM (Fig. 1E).

To sum up, nanomaterials exhibit extraordinary advantages in assisting detection of exo-miRNAs, they can be used as an efficient capture device, and can also be used as signal reporters based on enzyme-mimic activity, conductivity, and optical properties.

2.2. Isothermal signal amplification

Isothermal amplification technology receives widespread attention currently. It's not restricted by the complex operation and expensive equipment (Joshi et al., 2015; Zhao et al., 2015). Compared with traditional methods, isothermal amplification is more suitable in exo-miRNAs detection for clinical diagnosis. Most isothermal signal amplification methods can be classified as enzyme-assisted (Tang et al., 2020)or enzyme-free assisted (Liu et al., 2020b) signal amplification.

2.2.1. Enzyme-assisted isothermal amplification

The enzyme with high efficiency and specificity is an ideal choice for isothermal amplification. More and more enzymes that can be activated at constant temperature are selected for exo-miRNAs detection (Tang et al., 2020). In recent years, CRISPR-associated (Cas) proteins is found as an microbial adaptive immune system (Barrangou et al., 2007). In the presence of single-guide RNA (sgRNA), Cas9 proteins can precisely recognize protospacer adjacent motif (PAM) in the complementary double-strand DNA (dsDNA), and shear exogenous DNA specifically at this assigned point (Knott and Doudna 2018). Based on this principle, Wang et al. reported a CRISPR/Cas based approach for exo-miRNAs detection, which integrated CRISPR/Cas technology with rolling chain amplification (RCA) (Wang et al., 2020b). Firstly, specific padlock probe containing PAM hybridized with target miRNA and worked as trigger of RCA, HiFiTaq DNA ligase was used for linear extension during the amplification step (Fig. 2A). Consequently, the amplified long ssDNAs consists of a large number of repetitive target sequences and PAM structures were generated. The TaqMan probe hybridized with ssDNAs, Cas9 nuclease was activated and specifically cleaved to "turn on" fluorescence. Multicolor TaqMan probes that are complementary with distinct target miRNAs can be introduced to initiate multicolor detection.

Primer exchange reaction (PER), as a robust enzyme-assisted amplification strategy, can continuously amplify single strand which greatly improve the sensitivity of exo-miRNAs detection (Kishi et al., 2019). Kishi et al. introduced PER as synthesizing nascent single-stranded DNA via autonomous, specific cascade pathway (Kishi et al., 2018). Unlike RCA needs laborious step of circle synthesis and ligation. PER cascade only requires the participation of short oligonucleotide primer, multiple catalytic hairpins and polymerase for extension (Fig. 2B). When the right primer coexisted with matched hairpins, the long single-stranded DNA products could be grown and performed rapid signal amplification. Considering the superiority of PER, Li et al. designed an ultrasensitive electrochemical biosensor for exo-miRNAs detection based on PER and composite nanozyme (Li et al., 2020). The gated hairpins would transform to catalytic hairpins with the target miRNAs addition, which triggered the PER. Then, the primers would be extended as nascent single strand, numerous protector strands were capable to hybridize with PER products and release from capture probe (Fig. 2C). The nanozymes in the products hybridized with the capture probe, resulting in electrochemical signal changes. Under catalysis of nanozymes, a detection limit of 0.29 fM could be obtained.

2.2.2. Enzyme-free isothermal amplification

Compare with enzyme-assist amplification strategies, enzyme-free amplification strategies avoid the influences of the instability of enzymes. Enzyme-free isothermal amplification approaches usually drive target to cycling amplification, the two mostly used technologies are "DNA walker" design and SDR based on consecutive exposure of the toehold domain (Liu et al., 2020b). Zhang et al. constructed a radiometric electrochemical DNA biosensor based on bipedal DNA walkers for exo-miRNAs detection (Zhang et al., 2018). The two-footed DNA walkers were activated by target, and free to walk spontaneously along hairpin tracks, resulting in conformational changes. Subsequently, the reporter hairpins displaced DNA walkers and hybridized with capture probe, which produced significant increases in the ratio of target-dependent and target-independent signal (Fig. 3A). This bipedal DNA walkers method amplified signal in cascaded, so that the detection limit could reach 67 aM. Even though the aforementioned method greatly improves the sensitivity for exo-miRNAs detection, it still requires multiple DNA probes and relatively complicated strand displacement steps. To solve this problem, Luo et al. employed a "Y" shape structure formed by MB-signal strand and ferrocene (Fc) signal strand (Luo et al., 2020). In the presence of target, the MB-signal strand would transform into hairpin structure and dissociate Fc signal from electrode surface (Fig. 3B). The changes of conformation led to a strong signal of MB and a weak signal of Fc. The radiometric electrochemical biosensors showed good selectivity because of locked nucleic acid

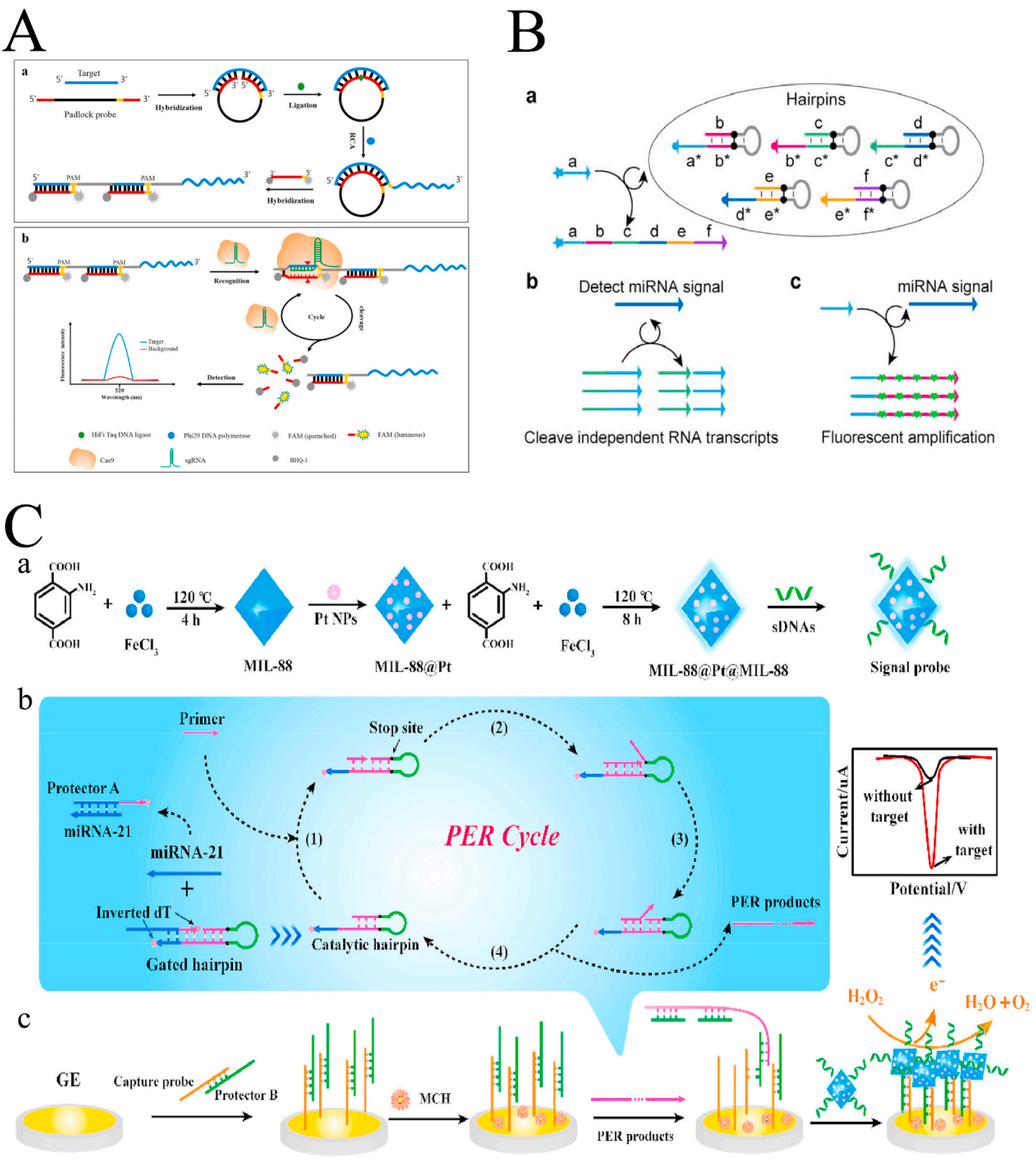

Fig. 2. (A) Rolling circular amplification (RCA)-assisted CRISPR/Cas9 cleavage (RACE) (Wang et al., 2020b). (B)PER synthesize nascent single-stranded DNA based on consecutive SDR (Kishi et al., 2018). (C) Ultrasensitive electrochemical biosensor based on PER and composite nanozyme (Li et al., 2020).

(LNA)-assisted toehold mediated strand displacement reaction (LSDR), which greatly improved the identification of mismatches and even single-base mismatches.

Hybridization chain reaction (HCR) is another efficient isothermal amplification method based on cross-opening of functional hairpins. When target miRNA was recognized, it would produce long double helix structures which were formed by hundreds of repeated units by hybridization of two DNA hairpins. He et al. designed hairpins carried DNAzyme subunits in 3′ and 5’ ends (He et al., 2018). In the presence of exo-miRNAs, the long DNA double helixes were formed, resulting numerous activated DNAzymes by two adjacent subunits (Fig. 3C). Then, the DNAzymes catalyzed the cleavage of fluorescence reporter probes, generating the fluorescence signal which can be used to quantify miRNAs in circulating exosomes. Guo et al. chose RuHex as signal

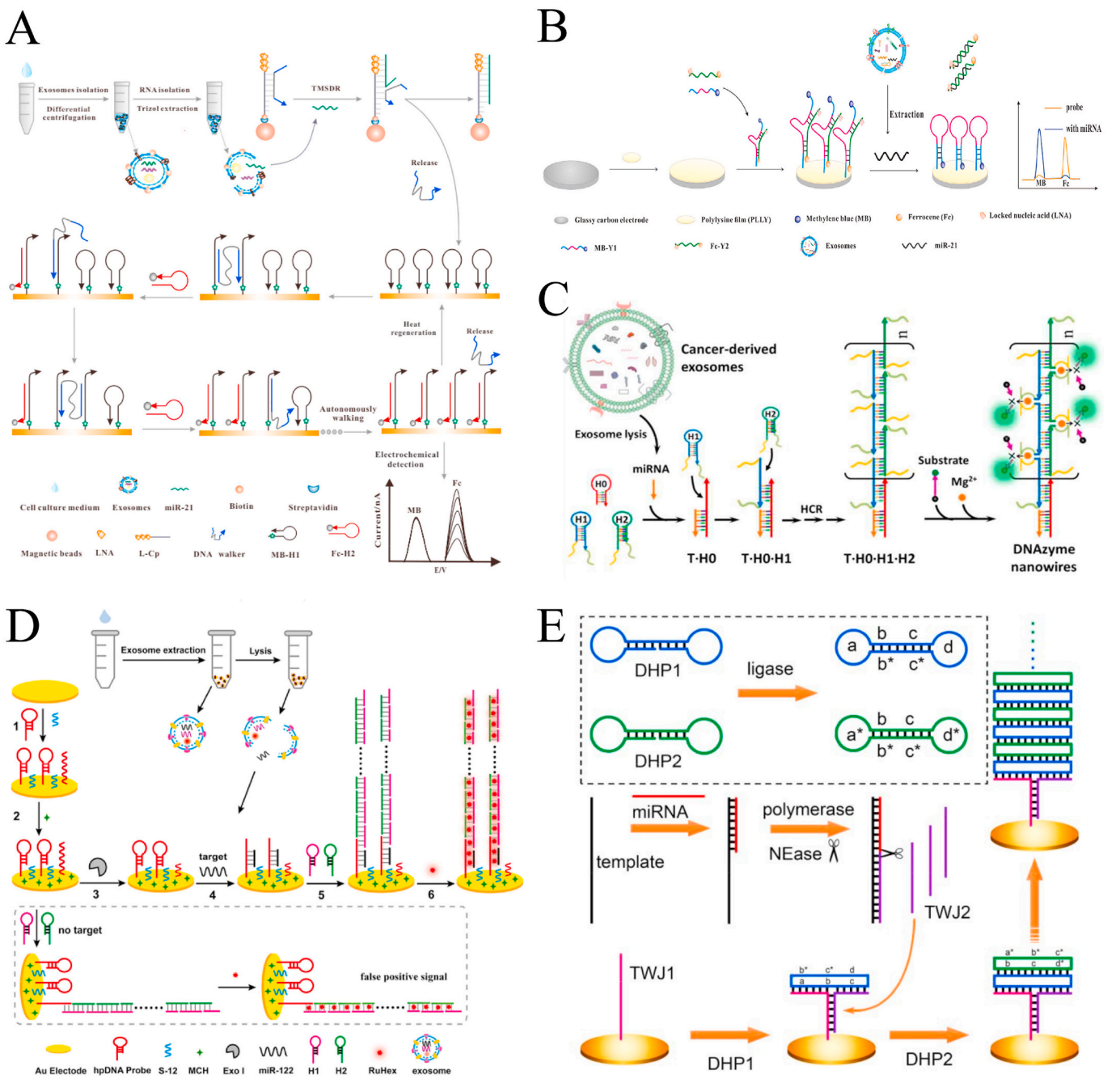

Fig. 3. (A) A radiometric electrochemical DNA biosensor based on bipedal DNA walkers for exo-miRNA-21 detection (Zhang et al., 2018).(B) Transformation of "Y" shape structure formed by MB and Fc signal strand for detecting exo-miRNAs (Luo et al., 2020).(C) Hairpins carried DNAzyme subunits in 3′ and 5′ ends formed polymer DNAzymes via HCR (He et al., 2018).(D) Electrochemical biosensor of exo-miRNAbased on HCR utilizing RuHex as signal reporter (Guo et al., 2020).(E) Dumbbell hybridization chain reaction (DHCR) to detect miRNAs (Miao and Tang 2020).

reporter (Guo et al., 2020). It was excellent electroactive molecule which showed a strong binding affinity to DNA duplexes (Fig. 3D). In this paper, RuHex directly bound to HCR products and produce intense electrochemical signal, which significantly simplify reaction process. Peng et al. proposed dumbbell hybridization chain reaction (DHCR) to detect miRNAs (Miao and Tang 2020). This method adopted dumbbell structured DNA probes instead of hairpin probes (Fig. 3E). Compare with the traditional HCR, it would produce closer DNA three-way junction structures. Based on the DHCR, this platform can detect down to 7.3 aM of exo-miRNAs.

2.3. *Microfluidic-assisted methods*

Microfluidics is an integrated platform for detecting micro-volume sample (Richards et al., 2017). The basic operating units including sample preparation, reaction, separation and detection are assembled integration. The microfluidic circuit can complete the analysis process in autonomous way (Cheung et al., 2018). Microfluidics have the advantages of portability, low consumption and easy to grasp. Combining fluorescence, electrochemical, chemical and other detection systems with microfluidics, the detection of exo-miRNAs can be more rapid and accurate (Goda et al., 2012). Ramshani et al. reported a surface acoustic wave (SAW) microfluidic platform (Ramshani et al., 2019). With fluid

flow in microfluidic channels, SAW lysed suspended exosomes directly by electric forces and released miRNAs (Fig. 4A). DNA probes were distributed on the ion-exchange membrane which is positively charged. Under the application of proper current, large amounts of exo-miRNAs captured by complimentary DNA probe would block the anions channel through holes of membrane, resulting in a measurable shift in voltage. This microfluidic device only needed 20 μL samples to detect exo-miRNAs. The whole reaction was completed in 30 min. Except for microfluidic chip, Zhou et al.,2020 designed a microfluidic strip based on lateral transverse flow and duplex-specific nuclease (DSN) assisted signals amplification (Zhou P 2020). After magnetic separation and lysis of exosome, miRNAs were released to bind with capture probes, generating DNA-RNA heteroduplexes (Fig. 4B). Capture probes were single-stranded DNAs carrying fluorescein isothiocyanate (FITC) and biotin. FITC and biotin would be separated after cleavage reaction of DSN. Lateral transverse flow test strips were used to analyze readout signal via binding antibody gold on the strip.

The analytical performance of the various strategies for exo-miRNA detection is summarized in Table 1.

3. Detection of exo-miRNA in single exosome

Although the above methods have fabulous sensitivity and specificity, they are time-consuming with the process of isolating exosomes and extracting exo-miRNAs. Moreover, there is no standard method for miRNA extraction, which highly affects the accuracy of detection. Thus, these methods are extremely limited in clinical application. Recently, more and more researchers pay attention at detecting exo-miRNAs in single exosome. Needing neither time-consuming nor costly extraction of miRNAs, those methods are simple, sensitive, accurate, and cost-effective, which are potent to be developed as non-invasive cancer diagnostic assays for clinical. It can also provide comprehensive information on the dynamics of exo-miRNAs. However, the complexity and interference of the environment around the exosomes make detection of exo-miRNAs in single exosome challengeable. Wherein, the major problem is the transport of detection systems. In this work, membrane fusion (Liu et al., 2020a), penetration (Chen et al. 2018, 2020; Cho et al., 2019; Lee et al., 2016; Wu et al., 2019) and membrane disposal (Wang et al., 2020a) used for exo-miRNAs detection in single exosome are mainly reviewed.

3.1. Membrane fusion

Membrane fusion plays an important role in the growth and development of life. Through fusion, two lipid bilayers are combined to complete a certain biological function. Therefore, it is a brilliant way to realize detection of exo-miRNAs by membrane fusion, which is also widely used in intracellular detection (Liu et al., 2020a). Gao et al. reported a rapid and sensitive strategy for exo-miRNAs detection based on virus mimicking fusogenic vesicle (Vir-FV) (Gao et al., 2019). In this strategy, highly efficient fusion between exosomes and Vir-FV was initiated due to the specially recognition of the fusogenic proteins (Fig. 5A). The molecular beacon (MB) probes enclosed in Vir-FV unfold by the target exo-miRNAs, achieving a great fluorescence signal. Using flow cytometry platform, the Vir-FV could distinguish tumor exosomes from normal exosomes by detecting exo-miRNAs in single exosome. The Vir-FV enabled high efficiency membrane fusion in human serum, which avoids the pre-isolation procedures. The whole reaction was completed within 2 h. Therefore, this platform revealed tremendous advancements toward raised efficiency of vesicle-fusion strategy.

Compared with the flow cytometry platform, the total internal reflection fluorescence microscopy (TIRF) makes membrane fusion more appropriate for biosensing. Wu et al. developed a tethered cationic lipoplex nanoparticles (tCLN) chip, which could detect exo-miRNAs (Wu et al., 2013). The tCLN biochip consisted of gold-coated cover glass and cationic lipoplex nanoparticles containing molecular sensing probes (Fig. 5B). In the presence of exosomes, the cationic lipoplex nanoparticles could fuse with the anionic exosomes and form nanoparticle complexes by electrostatic interaction. Then, the molecular beacons in cationic lipoplex nanoparticles hybridized with the target exo-miRNAs. Using total internal reflection fluorescence (TIRF) microscopy, the quantitative fluorescence signal of exo-miRNAs was detected. In this system, only 60 μL serum and 2.5 h was needed. Combined five exo-miRNAs (miR-21, miR-25, miR-155, miR-210, and miR-486), the tCLN assay distinguished normal controls from non-small cell lung cancer patients with AUC of 0.970 and sensitivity of 0.969. Such a platform was showed to work well for exo-miRNAs detection and clinical diagnosis.

3.2. Penetration

In order to maintain relative stability and activity in the constantly changing environment, membrane can absorb essential nutrients and excrete of metabolites (Lee et al., 2016). This selective property of the

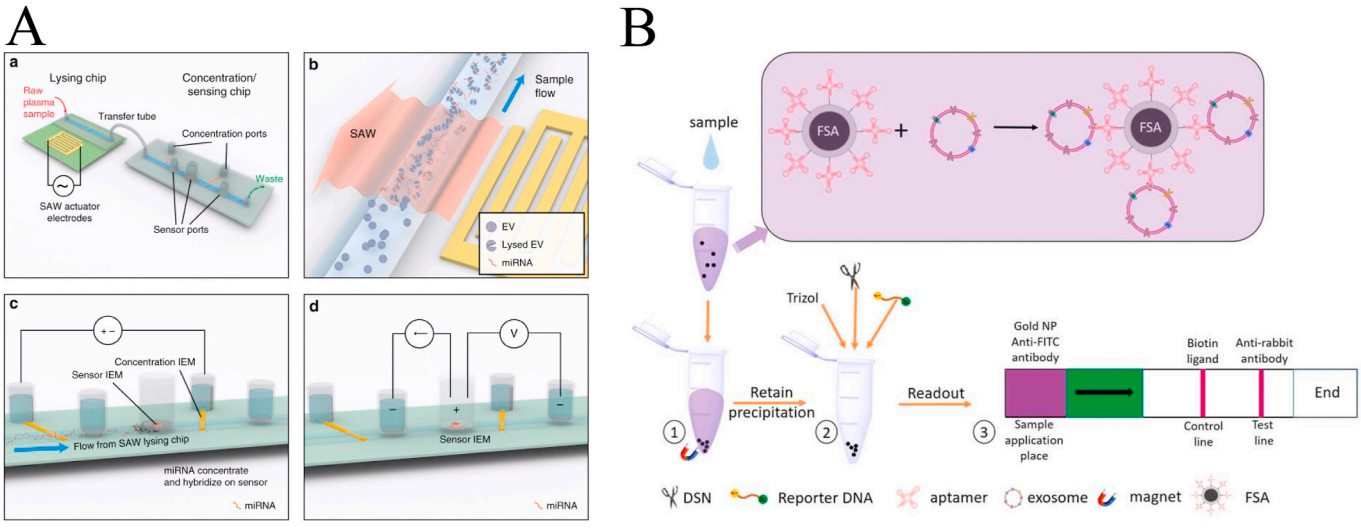

Fig. 4. (A) Surface acoustic wave (SAW) microfluidic platform for exo-miRNAs detection (Ramshani et al., 2019). (B) Microfluidic strip of exo-miRNAs detection based on lateral transverse flow and duplex-specific nuclease (DSN) assisted signals amplification (Zhou et al., 2020).

Table 1
Detection assays by exo-miRNA extraction.

Method	Technique	Target	Detection range (M)	Reaction Time (h)	LOD (M)	Clinical sample	Ref.
AuNP–Fe_2O_3NC	Electrochemical	miR-338–3p	10^{-16} to 10^{-9}	<2.5	10^{-16}	–	Masud et al. (2020)
SNA and PNA	Electrochemical	miR-21	10^{-16} to 10^{-9}	2	4.9×10^{-17}	Plasma	Liu et al. (2019)
AuNPs deposited as nanopillar	SERS	miR-21 miR-222 miR-200c	10^{-18} to 10^{-7}	–	10^{-18}	Serum	Lee et al. (2019)
Gold nanoprisms	LSPR	miR-10b	10^{-16} to 10^{-8}	12	8.3×10^{-18}	Plasma	Joshi et al. (2015)
ARANPs	SERS	miR-21	1.2×10^{-14} to 1.8×10^{-11}	3	5×10^{-15}	–	Ma et al. (2018)
Fe_3O_4 @Ag-DNA-Au@Ag@DTNB	SERS	miR-10b	3×10^{-18} to 1×10^{-12}	0.8	10^{-18}	Plasma	Pang et al. (2019)
AgNPs	Electrochemical	miR-21	10^{-15} to 2×10^{-10}	3.5	4×10^{-16}	Serum	Cheng et al. (2020)
DNA hydrogel	Imaging ellipsometry sensor (IES)	Let-7a miR-21 miR-375	2×10^{-16} to 5×10^{-13} 4×10^{-11} to 1×10^{-7} 1×10^{-14} to 7×10^{-10}	4-6	2×10^{-16} 4×10^{-11} 1×10^{-14}	Serum	Zou et al. (2020)
5,7-dinitro-2-sulfo-acridone (DSA) and carbon dots(CDs)	Fluorescence	miR-21	$<5 \times 10^{-13}$	2	3×10^{-15}	–	Xia et al. (2018)
RCA with CRISPR/Cas9	Fluorescence	miR-21	10^{-12} to 10^{-8}	3	9×10^{-14}	Plasma	Wang et al. (2020b)
RCA with G-quadruplex	Electrochemical	miR-21	10^{-14} to 10^{-8}	–	2.75×10^{-15}	Serum	Tang et al. (2020)
PER	Electrochemical	miR-21	10^{-15} to 10^{-9}	–	2.9×10^{-16}	Serum	Li et al. (2020)
versatile DNA nanosheet	Electrochemical	miR-21	10^{-16} to 10^{-9}	–	6.5×10^{-17}	Serum	Liu et al. (2020b)
Bipedal DNA walkers	Electrochemical	miR-21	10^{-16} to 10^{-13}	3	6.7×10^{-17}	Serum	Zhang et al. (2018)
LSDR	Electrochemical	miR-21	Not given	–	2.3×10^{-15}	–	Luo et al. (2020)
HCR with DNAzyme	Fluorescence	miR-21	10^{-11} to 10^{-8}	2	10^{-11}	Serum	He et al. (2018)
HCR with Exo I	Electrochemical	miR-122	10^{-16} to 10^{-7}	3	5.3×10^{-17}	–	Guo et al. (2020)
DHCR	Electrochemical	miRNAs	10^{-17} to 10^{-13}	3	7.3×10^{-18}	Serum	Miao and Tang (2020)
MOs with microfluidic channel	Fluorescence	miR-21	Not given	0.5	2.5×10^{-8}	–	Cheung et al. (2018)
SAW EV lysing with concentration and sensing microfluidic chip	Electrochemical	miR-21	10^{-12} to 10^{-9}	0.5	10^{-15}	Plasma	Ramshani et al. (2019)
Strip based on lateral transverse flow	Fluorescence	miR-205	$<10^{-7}$	–	7.76×10^{-12}	Urine	(Zhou P 2020)

membrane is called selective permeability (Cho et al., 2019). This is one of the most basic functions of the exosome membrane (Chen et al., 2018). Thus, some smaller molecules with rigidly can penetrate the membrane freely, such as molecular beacons, Au Nanoparticles and DNA tetrahedrons.

AuNPs, as a nano-size material, provide an appropriate micro-environment for target recognition (Chen et al., 2020). Their large area is important for loading signal amplification component and enabling high efficiency of fluorescence resonance energy transfer (FRET) (Wu et al., 2019). Zhai et al. designed an Au nanoflare probe for detecting exo-miRNA encased in plasma directly (Zhai et al., 2018). This probe consisted of three portions, including 13 nm Au nanoparticle, capture DNA and report DNA (Fig. 6A). The capture DNA was fixed on the surface of the Au nanoparticle by S–Au bond. Cy3 modified report DNA hybridized to the capture DNA led to the Cy3 quenched by the Au nanoparticle by FRET. In the presence of the exosomes, the Au probe entered the exosomes by membrane penetration and specially hybridized to the target exo-miRNAs. Then, the report DNA was released by toe-hold mediated strand displacement, which generated quantifiable fluorescent signals. Using fluorescence spectrometer, this system can detect spiked exosomes in normal human plasma samples, and an outstanding recovery rate of around 100% was achieved. Compared with the fluorescence spectrometer, the thermophoretic platform using Au nanoprobe can obtain better sensitivity. Sun et al. adopted a similar Au nanoprobe for exo-miRNA detection (Zhao et al., 2020). Under the advantage of thermophoretic platform, it can detect exo-miRNA down to 0.36 fM with a liner range from 0.17 to 170 fM (Fig. 6B).

3.3. Membrane disposal

Streptolysin O (SLO) is a Piercing bacterial toxin that has been used as a reversible membrane permeabilization tool for live cell imaging of miRNAs using molecular beacon probes (MBs) (Wang et al., 2020a). Compared with other delivery methods, such as microinjection and electroporation, this method is simpler, more efficient and non-toxic, and will not cause severe damage to cells. Lee et al. proposed the strategy of using SLO and MBs to detect exo-miRNAs (Lee et al., 2015). SLO could bind to cholesterol on the plasma membrane of the exosomes and oligomerizes to form pores that were less than 30 nm in diameter, allowing the influx of MBs (Fig. 7A). The hybridization of the target miRNAs and MBs in the exosomes opened the hairpin and physically separated the reporter molecule from the quencher molecule, allowing a fluorescent signal to be emitted upon excitation. Using this method, miR-21 in tumor cell-derived exosomes could be detected within 1 h.

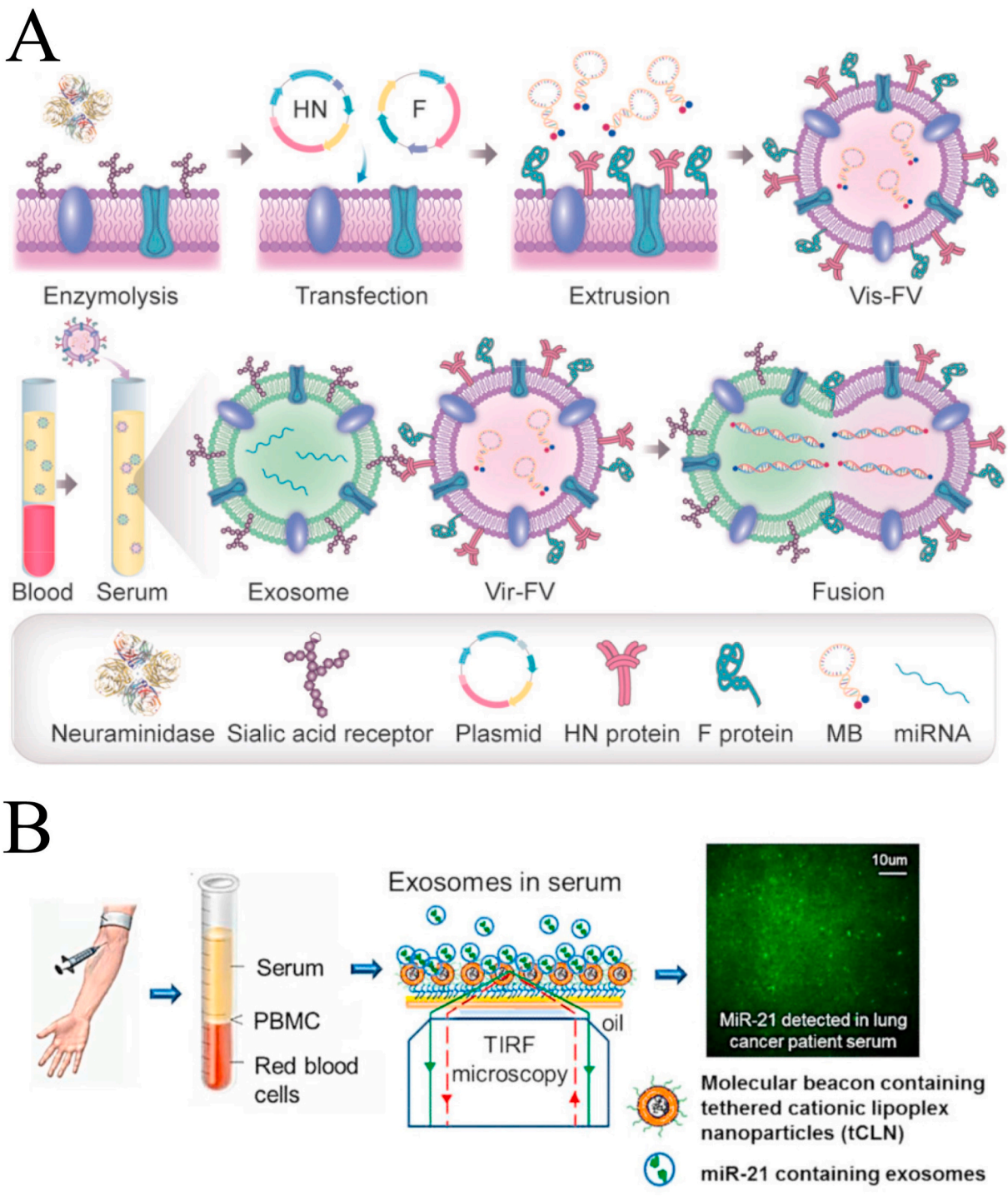

Fig. 5. (A) Exo-miRNAs detection based on virus mimicking fusogenic vesicle (Vir-FV) (Gao et al., 2019). (B)Tethered cationic lipoplex nanoparticles (tCLN) chip for exo-miRNAs detection (Wu et al., 2013).

The direct detection technology using SLO and MB would provide a new idea for cancer diagnosis and prognosis. In order to improve the detection sensitivity, He et al. established a single vesicle imaging analysis method based on total internal reflection fluorescence (TIRF) to directly display and quantify the content of exo-miRNAs in serum samples (He et al., 2019). Firstly, the inactivated lytic DNAzyme and the fluorescence quenching substrates were co-delivery to the nano-sized exosomes treated with SLO. Then, the target miRNA activated the substrates to generate a catalytic cleavage reaction, which could amplify the readout of the fluorescence signal (Fig. 7B). The LOD of the assay was estimated to be 378 copies μL^{-1}, which is equivalent to qRT-PCR.

The analytical performance of the various strategies for exo-miRNA detection in single exosome is summarized in Table 2.

4. Summary and outlook

In this review, we have discussed progress made toward engineering biosensors for exo-miRNA detection in various samples. Several amplification strategies were summarized, including nanomaterials, isothermal amplification and microfluidic. We also described the

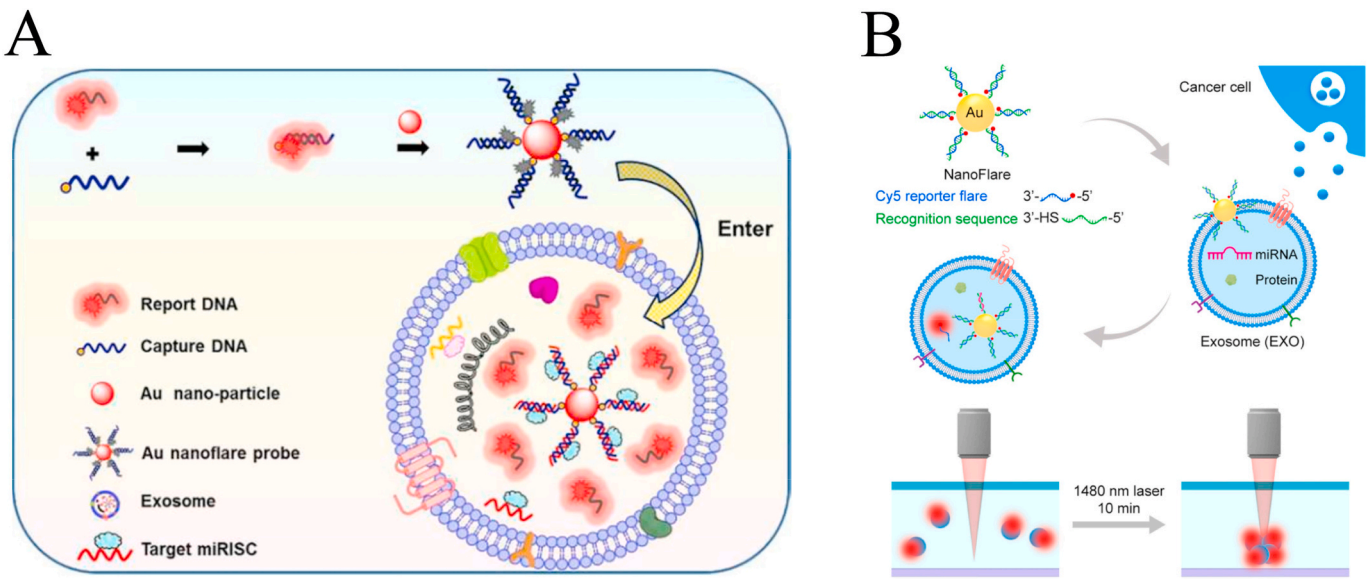

Fig. 6. (A) Au nanoflare probes for exo-miRNA detection (Zhai et al., 2018). (B) Au nanoprobe for exo-miRNA detection with thermophoretic platform (Zhao et al., 2020).

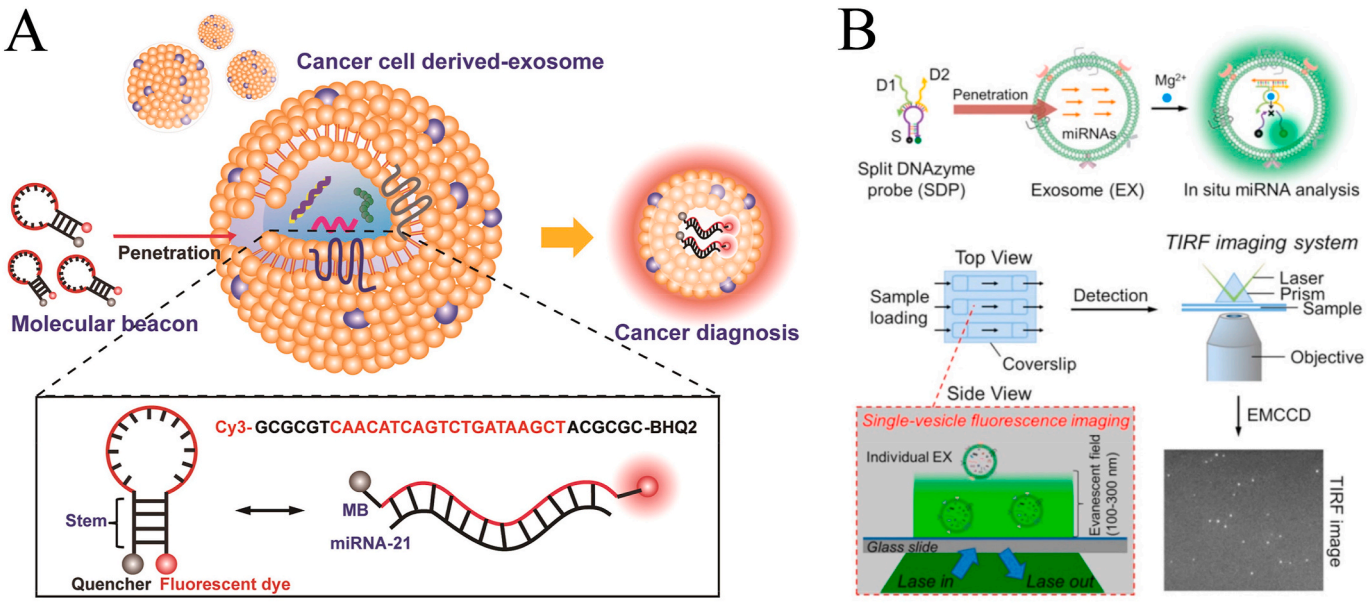

Fig. 7. (A) Strategy of SLO and MBs used to detect exo-miRNAs (Lee et al., 2015).(B) Single vesicle imaging analysis method based on total internal reflection fluorescence (TIRF) to directly display and quantify the exo-miRNAs (He et al., 2019).

development of detecting exo-miRNA in single exosome. We compare and contrast the two types of exo-miRNA detection methods (Table 3). Each type has advantages and disadvantages, this table should assist in identifying features that can be most readily used for different sensor modalities and applications. Despite the advantages and progress made, this field is still an active domain that needs further development.

4.1. Sample pretreatment

To advance the clinical applications of exo-miRNAs assays, a suitable pretreatment on samples improves the comparability of results between instruments and institutes (Clayton et al., 2019). Exosomes in cell culture medium and serum may be isolated using ultracentrifugation (UC) and commercial kit ExoQuick. The low recovery of UC limits the sensitivity of the biosensor. ExoQuick leads to high contamination by free miRNA. To obtain the reliability and repeatability of exo-miRNA biosensor, a suitable sample pretreatment is needed firstly.

4.2. Detection exo-miRNA in single exosome

The exo-miRNA detection in single exosome offer intact information in individual exosome and avoid the miRNA extraction, which is important for clinical application. Membrane fusion shows great potential for exo-miRNA detection in this field. Since membrane fusion can be used to transmit various amplifiers efficiently for exo-miRNA nondestructive detection, a whole array of new research for exo-miRNAs detection can be explored and a more efficient membrane will be proposed in the future. At present, few biosensors possess spatio-temporal control capacity of the detection system's activity, and some background signals may be generated due to inappropriate initiation

Table 2
Detection of exo-miRNA in single exosome.

Methods	Technique	Delivery	Target	Detection range (particles/μL)	Reaction time (h)	Clinical sample	Ref.
Vir-FV	fluorescence	Membrane fusion	miR-21	–	2	Serum	Gao et al. (2019)
tCLN-MB biochip	TIFR	Membrane fusion	miR-21	–	2	Serum	Wu et al. (2013)
tCLN-MB biochip	TIFR	Membrane fusion	miR-21, miR-25, miR-155, miR-210, miR-486	–	~2.5	Serum	Liu et al. (2020a)
MB	SMLM	Penetration	miR-21, miR-31	–	2	–	Chen et al. (2018)
MB	fluorescence	Penetration	miR-21, miR-27a, miR-375	–	1	Serum	Lee et al. (2016)
MB	fluorescence	Penetration	miR-21	10^7 to 10^9	4	–	Cho et al. (2019)
fLIGHT nanoprobe	fluorescence	Penetration	miR-21	–	4	Plasma	Chen et al. (2020)
Au nanoflare	fluorescence	Penetration	miR-1246	0.2×10^8 to 5×10^8	4	Plasma	Zhai et al. (2018)
Au nanoflare	thermophoretic accumulation	Penetration	miR-375	10^3 to 10^7	2	Serum	Zhao et al. (2020)
CHCR-DNAzyme	fluorescence	Electroporation	miR-21	–	5	Serum	Wu et al. (2019)
MB	fluorescence	SLO	miR-21	0 to 6×10^7	1-2	Serum	Lee et al. (2015)
MB	TIFR	SLO	miR-21, miR-221	3×10^4 to 3×10^7	1	Serum	He et al. (2019)
MB	fluorescence	SLO	miR-21, miR-27a and miR-375	–	0.5	Serum	Wang et al. (2020a)

Table 3
Comparison of massive study and individual study.

category	massive study	individual study
detection method	electrochemical/ fluorescence spectrometer/ SERS/LSPR	fluorescence spectrometer/ TRIF/thermophoretic accumulation
RNA extraction	extraction	extraction-free
total reaction time (h)	4.5-16	1-5
operation	relatively complex	simple
amplification type	multiple	single
efficiency	good	moderate
specificity	moderate	good

during the transport process. Therefore, a light-controlled biosensor should be proposed to address this issue in exo-miRNA detection with enhanced spatial and temporal precision.

4.3. Clinical transformation

With the advances of biosensors for exo-miRNA detection, various strategies have been designed and explored for clinical diagnostics. However, a clear "bench to bedside" translation is still missing may be due to the well-developed PCR commercial infrastructure. Combined exo-miRNA sensors with well-accepted devices (such as routinely used personal glucose meter) may be the solution. It will greatly advance clinical translation by benefiting from many product developments. In addition, exo-miRNA expression profile analysis should be developed for more clinically relevant targets, which will certainly attract the attention of clinicians. Machine learning can widen the application of exo-miRNA expression profile analysis in biosensing.

4.4. Concluding remarks

Considering the current research in understanding the biological functions of exo-miRNA in biochemical and clinical research, the continued advance of biosensor for exo-miRNA is very important. Even though exo-miRNA biosensors have been developed in a short time, they have emerged as one of the most powerful roles to make a major impact on scientific research.

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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