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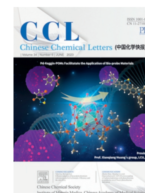

Recent advancements in DNA nanotechnology-enabled extracellular vesicles detection and diagnosis: A mini review

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ABSTRACT

Extracellular vesicles (EVs) are cell-derived nanosized vesicles widely recognized for their critical roles in various pathophysiological processes. Molecular analysis of EVs is currently being considered an emerging tool for diseases diagnosis. However, the small size and heterogeneity of EVs has staggered the EVs research for diseases diagnosis. DNA nanotechnology enables self-assembly of versatile DNA nanostructures and has shown enormous potential in assisting EVs biosensing. In this review, we briefly introduce the recent advances in DNA nanotechnology approaches for EVs detection. The approaches were categorized based on the dimension of DNA nanostructures. We provide critical evaluation of these approaches, and summarize the pros and cons of specific methods. Further, we discuss the challenges and future perspectives in this field.

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1. Introduction

Extracellular vesicles (EVs) are membrane-bound nanosized vesicles released from cells. EVs were discovered in 1980s in sheep reticulocytes samples, initially the secretion of EVs was simply considered a mechanism for eliminating unnecessary compounds from the cell [1]. However, in 1990s, researchers started to find that EVs might participate in intercellular communication, especially in immune regulation [2]. Later in 2007, this fact was strongly established that EVs have role in exchanging information between cells [3]. Since then, EVs research has witnessed exponential increase not only in the knowledge of the classes and characteristics of EVs but also about the important roles of EVs during a plethora of pathophysiological conditions. As EVs are a heterogeneous group containing various subtypes defined based on their biogenesis, size, and composition (*e.g.*, exosomes, microvesicles, apoptotic bodies, and oncosomes), there have been controversies about nomenclatures during the development of EVs science. To help convergence to a common language and comparable experimental settings and results, the International Society for Extracellular Vesicles (ISEV) published a list of minimal information guidelines covering the main features of vesicles and common terminology, isolation, characterization and functional studies [4,5]. They strongly suggested to use the generic term “EV” unless the authors can establish specific markers of the subcellular origin or the EVs are caught in the act of release by live imaging techniques to be called otherwise. With the concerted efforts of ISEV and many researchers in the field, standardization indices for EVs research are gradually establishing. It is now widely acknowledged that EVs are versatile carriers having particular nucleic acid, proteins and lipid compositions. The nature of EVs contents depends on the type and physiological status of their donor cells. Once released into the extracellular space, EVs can deliver their contents to recipient cells such as dendritic cells and T lymphocytes to elicit functional responses [6].

The revolutionary progress in understanding the biology of EVs has led to the emerging application of EVs as biomarkers to diagnosis and monitor the progression of various human disorders including cancer and neurodegenerative diseases [7]. To analyze EVs compositions and provide useful tools for clinical diagnosis, various biosensing platforms have been developed [8]. However, the small size and heterogeneity of EVs lead to immense challenges for EVs detection. Classic characterization equipment such as nanoparticle tracking analysis (NTA)-based platforms, resistive pulse sensing (RPS) and atomic force microscope (AFM) can only provide partial information about the physical properties of EVs. Conventional biochemical analysis techniques such as enzyme-linked im-

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Table 1
EVs biomarkers in a sample of human disease.

Disease	EVs biomarker	EVs source	Ref.
Cancer	Density of EVs, miRNA (miR-185, miR-21, *etc.*), lncRNA (CEBPA-AS1, HOTTIP, *etc.*), protein (BARHL2, MT1-MMP, *etc.*)	Plasma, serum, ascites, gastric juice, saliva, breast milk	[19,22–24,26]
Cardiovascular disease	Density of EVs, miRNA (miRNA-126–3p, miRNA-208a, *etc.*), proteins (CD144, CD31, *etc.*)	Whole blood, serum, plasma	[28]
Neurodegenerative diseases	Protein (Aβ1–42, T-Tau, *etc.*), miRNA (miR-331–5p, miR-223–5p, *etc.*)	Serum, plasma, cerebro-spinal fluid, saliva, urine	[17,29,30]
Type 2 diabetes	Density of EVs, miRNA (miR-23a, miR-192, *etc.*), mRNA	Serum, plasma, urine, intestinal microbes	[31]
Autoimmunity disease	miRNA (miR-146a, miR-155 *etc.*), protein (CD41, citrullinated proteins, *etc.*)	Serum, plasma, synovial fluid, urine	[33]
Sepsis and ARDS	ncRNA (Lnc-p21, lnc-TUG1, *etc.*), protein (Laccase, A2MG, *etc.*)	Bronchoalveolar lavage fluid, serum, plasma	[34]

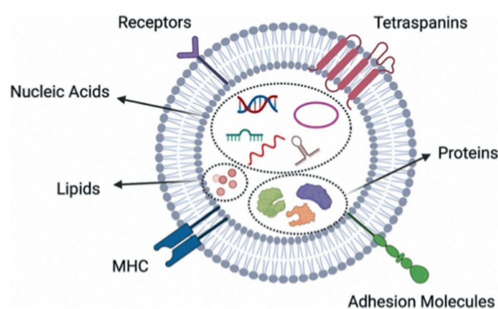

Fig. 1. Extracellular vesicle structure and content. Copied with permission [18]. Copyright 2022, Elsevier.

munosorbent assay (ELISA), reverse-transcription quantitative polymerase chain reaction (PCR) [9,10] and mass spectroscopy suffer from several limitations, for instances, low sensitivity, complex pre-processing, time consuming operations requiring skilled operators and dedicated spaces. To alleviate these limitations there is an urgent need to develop EVs detection methods with improved sensitivity, convenience and flexibility.

DNA nanotechnology has grown rapidly in the past decades. DNA nanostructures are actively explored for developing sensors due to their unique properties such as predictability and programmability due to intermolecular interactions (Watson-Crick base pairing), convenient chemistry synthesis and modification, controllable surface chemistry and dynamic function, prototype for numerous structures shape at one, two or three dimensions [11,12]. In this review, we will summarize the recent advances in DNA nanotechnology-assisted EVs biosensing. First, we will provide overview of the widely accepted diagnostic potential of EVs as an ideal new generation tool for clinical diagnosis of diseases. Then provide a brief introduction of the origin and development of DNA nanotechnology. Following this we will discuss the current strategies of applying DNA nanostructures into EVs detection highlighting their performance, pros and cons. Finally, we will discuss the current achievements in this area as well as discuss the challenges and opportunities laying ahead.

2. Diagnostic application of extracellular vesicles

Biomarker can provide critical information to help identify a particular disease and assess the status or extent of the disease. An ideal biomarker should be stable, easy to obtain with minimum discomfort or risk to patients, detectable in the early stage of the disease and changeable with intervention. Numerous studies have proved the application prospects of EVs to serve as ideal biomarkers for the diagnosis of multiple human disorders (Table 1). Firstly, EVs exhibit unique compositional characteristics, high abundance and physiochemical stability compared with nucleic acid biomarkers [13,14]. They carry various bioactive molecules involved in pathophysiological regulation (Fig. 1). Nucleic acid including genomic DNA, miRNA and mRNA is an essential part of EVs-contained bioactive molecules. It has been reported that cellular RNAs are selectively loaded into EVs depend on endogenous loading processes such as endosomal pathways and miRNA pathways. As for the origins of DNA content in EVs, very little is known at present. One potential mechanism involves early endosomes maturing into late endosomes and accumulating intraluminal vesicles [4]. EVs population and its composition can reflect the inflammatory or immune activated state, disease conditions, regeneration process and response to intentional actions [15]. Secondly, EVs can be isolated from biofluids such as saliva, urine and blood with minimal invasiveness. Thirdly, many studies vouch for EVs utility in early diagnosis of diseases [16,17].

Numerous shreds of evidence have pointed out the potential of EVs in improving the diagnosis and prognosis of various diseases, here we only list a few of them. The majority of studies on EVs as diagnostic and prognostic biomarkers are focused on cancers. By promoting cancer cells growth, invasion and metastasis, enhancing angiogenesis, EVs play a significant important role in tumorigenesis [19,20]. Differential expression of EVs proteins and their cargo are noted in various types of cancer [21] including gastric cancer [22], bladder cancer [23], breast cancer [24,25] and ovarian cancer [26]. In cardiovascular disease [27], since cellular activation and apoptosis can trigger the release of EVs, patients with systemic endothelial damage show significantly increased levels of circulating endothelial cell derived EVs [28]. More recently, the role of EVs in the initiation and progression of neurodegenerative diseases has been validated. As EVs can cross the blood-brain barrier, they can give a fine imprint of central nervous system associated disease pathologies including Alzheimer's disease [29], Parkinson's disease [30], Huntington's disease and amyotrophic lateral sclerosis [17]. Moreover, EVs can also serve as biomarkers in type 2 diabetes [31,32], autoimmunity disease [33], sepsis and acute respiratory distress syndrome (ARDS) [34].

3. DNA nanostructures assisted biosensors

3.1. Brief introduction to DNA nanotechnology

Based on Watson-crick base pairing, Seeman used four synthetic DNA strands to build the first immobile Holiday junction with the ability to self-assemble into two-dimensional (2D) lattices [35]. Since then, DNA nanotechnology has grown rapidly in the last two decades. The main strategies to build DNA nanostructure includes tile-based bottom-up assembly using robust DNA tiles such as Holiday junction, six-helix bundles and triangular motif to construct higher dimensional nanostructures, origami assembly using long single-stranded DNA as scaffold to construct 2D and three-dimensional (3D) shapes, nanoparticle-templated assembly employing inorganic or organic nanoparticles as a core to organize DNA [36], hybrid of DNA tiles, origamis, metal-DNA junctions and nanoparticle-templated DNA nanostructures [37], and other methods such as rolling circle amplifica-

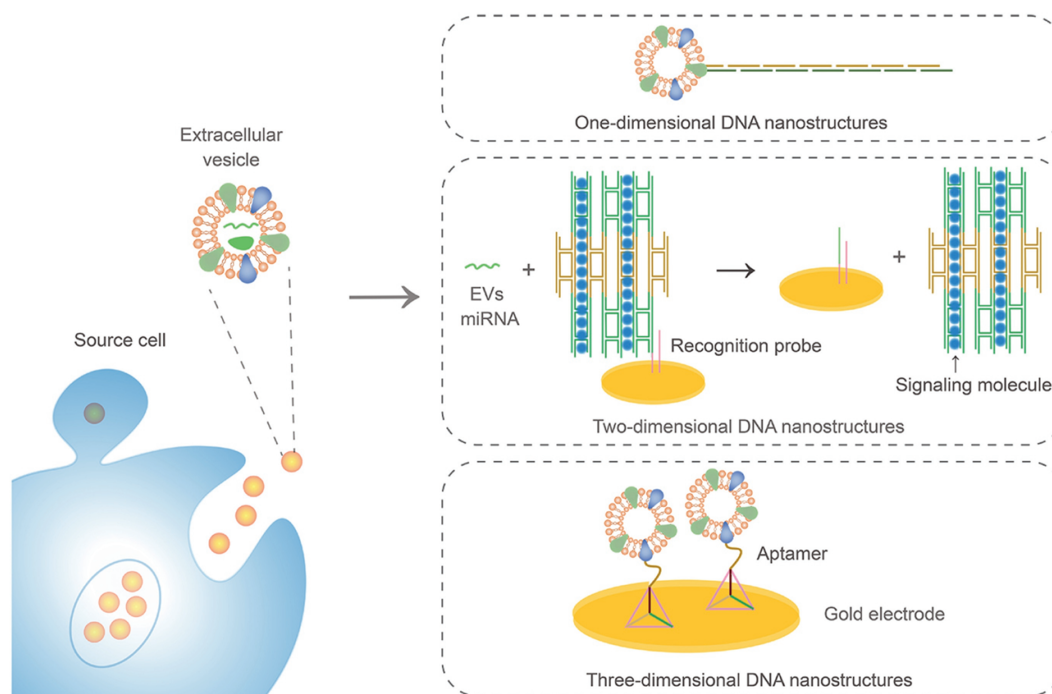

Fig. 2. The participation of DNA nanotechnology in the detection of EVs. DNA nanostructures are categorized into three types: one-dimensional DNA nanostructures, two-dimensional DNA nanostructures and three-dimensional DNA nanostructures.

tion (RCA) using circular DNA templates to produce long strand DNA [11]. Majority of the above mentioned DNA nanostructures have been applied to improve the performance of EVs biosensors. Based on the type of DNA nanostructures the following categories can be made: (i) one-dimensional DNA nanostructures, (ii) two-dimensional DNA nanostructures and (iii) three-dimensional DNA nanostructures (Fig. 2). We will review current research status in this field and discuss the pros and cons of specific methods. Comprehensive metrics are summarized in a table for easier reading (Table 2).

3.2. One-dimensional DNA nanostructures

A strategy of DNA biosensors relies on the transformation of DNA nanostructures caused by the presence of target analytes. Aptamers, stem-loop structures and other functional DNAs capable of recognizing the targets directly are commonly used [38,39]. Wherein, stem-loop structures [40–42], DNA duplex [43–46] and DNA junctions [47] are most frequently used to detect exosomal miRNAs. Aptamers are single stranded oligonucleotides with high affinity towards various targets including microorganisms, proteins and ions [48]. Aptamers are generated by an *in vitro* process named systematic evolution of ligands by exponential enrichment (SELEX) [49,50]. Compared to antibodies, aptamers are less pricy, highly stable and amenable to chemical modifications. Zhang and co-workers formed a hybridization complex using EV membrane protein CD63-specific aptamer and two complementary sequences and conjugated the complex onto magnetic beads (Fig. 3A) [51], which have been widely used for magnetic separation in biosensors [52,53]. The presence of EVs broke up the complex and resulted in the etching of gold nanobipyramid@MnO_2 nanosheet nanostructures, accompanied by the change of ultraviolet-visible spectroscopy absorption spectra. The assay was an absorbance-based readout with narrow dynamic range (8.5×10^2 to 8.5×10^4 particles/μL) as dictated by the optical density. Which could lead to a repeat run of the entire detection procedure as some samples might be out of range.

Besides recognition element [54,55], rationally designed aptamers can also serve as signal transduction elements and amplifier of output signals in biosensors [56]. Hold the advantage of easy to design and controllable abilities [57], aptamer-assisted recycling process [58–60], nonenzymatic nucleic acid amplification techniques such as hybridization chain reaction (HCR) [61–63], dynamic DNA strand displacement [64,65] such as DNA logic gates and DNA walker [66–69] have attracted huge attention in EVs biosensing. Yu and co-workers designed a target triggered DNA walker for the quantification of tumor-derived EVs (Fig. 3B) [70]. The DNA walker was constructed using magnetic bead (MB) modified with CD63-specific aptamers and a swing arm. Target EVs could bind with MB and swing arm simultaneously through the recognition between aptamer and EVs surface protein, resulting in the close proximity effect between DNAzyme on the swing arm and the substrates on the MB, which led to the movements of the DNA walker through alternate hybridization and cleavage. Due to their flexibility, compact size and low-cost [71–73], electrochemical biosensors are widely chosen in EVs detection. Liu and co-workers designed an electrochemical biosensor based on the recognition of EVs by Zr-metal-organic framework (Zr-MOF) and aptamer to trigger the HCR for signal amplification [74]. In their study, the binding between CD63-specifice aptamer and EVs captured by Zr-MOF could initiate the HCR amplification and produce DNA nanowires containing hemin/G-quadruples DNAzymes to catalyze the oxidation of substrate. The assay was cheap, convenient and ultrasensitive. However, the target (CD63) they used lacks specificity and clinical verification is not included in their study.

Development in isothermal amplification technology have led to the exploration of this approach. Rolling-circle amplification (RCA) can generate long strand DNAs with repeated unit containing sequences complementary to the circular DNA template, providing a convenient and efficient way for the construction of DNA nanostructures [75–77]. Our group's research largely focused on the isolation and subsequent molecular analysis of EVs in the last decade. We are particularly interested in the ultrasensitive detection meth-

Table 2
A sample of DNA nanostructures used in the detection of EVs.

Detection method	Mechanism	Limit of detection	Features	Drawbacks	Ref.
1D DNA nanostructures					
Nanowire	HRP catalyzed oxidation & HCR assisted signal amplification	96 particles/μL	Ultrasensitive	Cumbersome detection, complex preparation	[61]
	G-quadruplex enhanced fluorescence & strand replacement reaction assisted signal amplification	3.4×10^5 particles/μL	Without enzyme, label-free, simple detection	Low sensitivity, narrow dynamic range	[65]
	G-quadruplex enhanced fluorescence & TdT assisted signal amplification	3.6×10^2 particles/μL	Without separation steps, heterogeneity identification, stable in complex medium	Lack of validation with clinical samples	[87]
DNA walker	Target EVs triggered and restriction endonuclease powered DNA motor process	8.2 particles/μL	Ultrasensitive, stable in complex biological samples	Needs for enzymes, construction of complex DNA motor	[70]
DNA logic gate	Target triggered cyclic enzymatic signal amplification	9.2×10^2 particles/μL	Multiple validations, multi-protein detection	Cumbersome and time-costing detection	[95]
2D DNA nanostructures					
DNA dendrimer	Fluorescent dye & Branched RCA assisted signal amplification	42.7 particles/μL	Simple design	Compete washing steps, unstable in complex biological samples	[78]
	FAM-labeled DNA strand & Hairpin DNA cascade reaction assisted signal amplification	1.16×10^3 particles/μL	Low-cost, without enzyme	Low sensitivity, Cumbersome detection	[100]
	HRP catalyzed oxidation & HRCA assisted signal amplification	0.334 particles/μL	Ultrasensitive, stable in complex medium	Time-costing, complex preparation	[102]
	Target induced fluorescence recovery & HCR assisted signal amplification	Not reported	Real-time monitoring, without enzyme, living cell compatible, point of care potential	Complex preparation, equipment requirements	[104]
DNA nanosheet	G-quadruplex/hemin DNAzyme catalyzed oxidation & ʟ-DCDR assisted signal amplification	65 amol/L (EVs miRNA)	Ultrasensitive, multiple validations, without enzyme	Complex preparation, time-costing detection	[105]
3D DNA nanostructures					
DNA tetrahedra	Ferricyanide-ferrocyanide redox couple for signal generation & DNA tetrahedra-assisted target capture	3.96×10^2 particles/μL	Efficient target capture, enhanced detection range	Lack of validation with clinical samples	[109]
DNA nanocube	Fluorescence resonance energy transfer & fluorescence resonance energy transfer assisted signal amplification	9.8×10^4 particles/μL	Living cell compatible, *in situ* imaging	Low sensitivity, narrow dynamic range	[115]
DNA nanoflower	RCA products undergo structural change to generate localized amplified fluorescent signals	10 particles/μL	Integrated logic, ultrasensitive, multi-protein profiling, multiple validations, stable in complex biological samples	Complex preparation	[117]
	HRP catalyzed oxidation & RCA produced nanoflower for target recognition	3.32×10^3 particles/μL	Without enzyme, easily observed with the naked eye	Complex preparation, time-costing	[119]
Hyperbranched DNA superstructure	Dual-aptamer recognition system to isolate EVs & DNA tetrahedron-based hyperbranched HCR products to load Ru(III)	30 particles/μL	Ultrasensitive, integrating EVs isolation and analysis	Complex preparation, small sample size	[121]
3D DNA machine	Target triggered amplification reaction to produce ratiometric to fluorescence signal	9.9 particles/μL	Multiple validations, heterogeneity identification, multi-protein profiling, ultrasensitive, potential in screening early urinary diseases	Complex preparation	[122]

ods based on RCA [78–81]. Recently, we used probe-based exponential RCA to quantify cancer cell-derived EVs. We designed an aptamer-primer sequence to trigger RCA at the presence of target EVs [79]. The proposed method was able to distinguish serum samples from cancer patients and healthy donors. In a parallel study, Gao and co-workers developed a RCA-assisted flow cytometry approach to profile EVs surface proteins (Fig. 3C) [82]. The aptamer-primer sequence bound with EVs could trigger RCA reaction, providing repeated DNA sequences for fluorescent probe hybridization. Other isothermal amplification techniques including terminal deoxynucleotidyl transferase [TdT] dependent amplification [83,84] and dual-cycle amplification [85] have also been used for EVs detection. Due to their fast response, multiple analysis, simple operation and instrument [86], fluorescent biosensors were widely chosen in these detection methods. Wang and co-workers reported an aptamer-coated liposome complex for the detection and profiling of EVs based on TdT-mediated signal amplification [87]. CD63-specific aptamers immobilized on the surface of liposomes tended to bind with target EVs and the resulting aptamer-EV complex could trigger polymerization reaction through the access to TdT for signal amplification.

The phenotype heterogeneity of EVs, along with the slight differences in surface protein expression levels between disease-associated EVs and normal EVs makes the detection based on only one biomarker unreliable [88,89]; which may limit their potential for clinical translation. Thus, some approaches have been designed

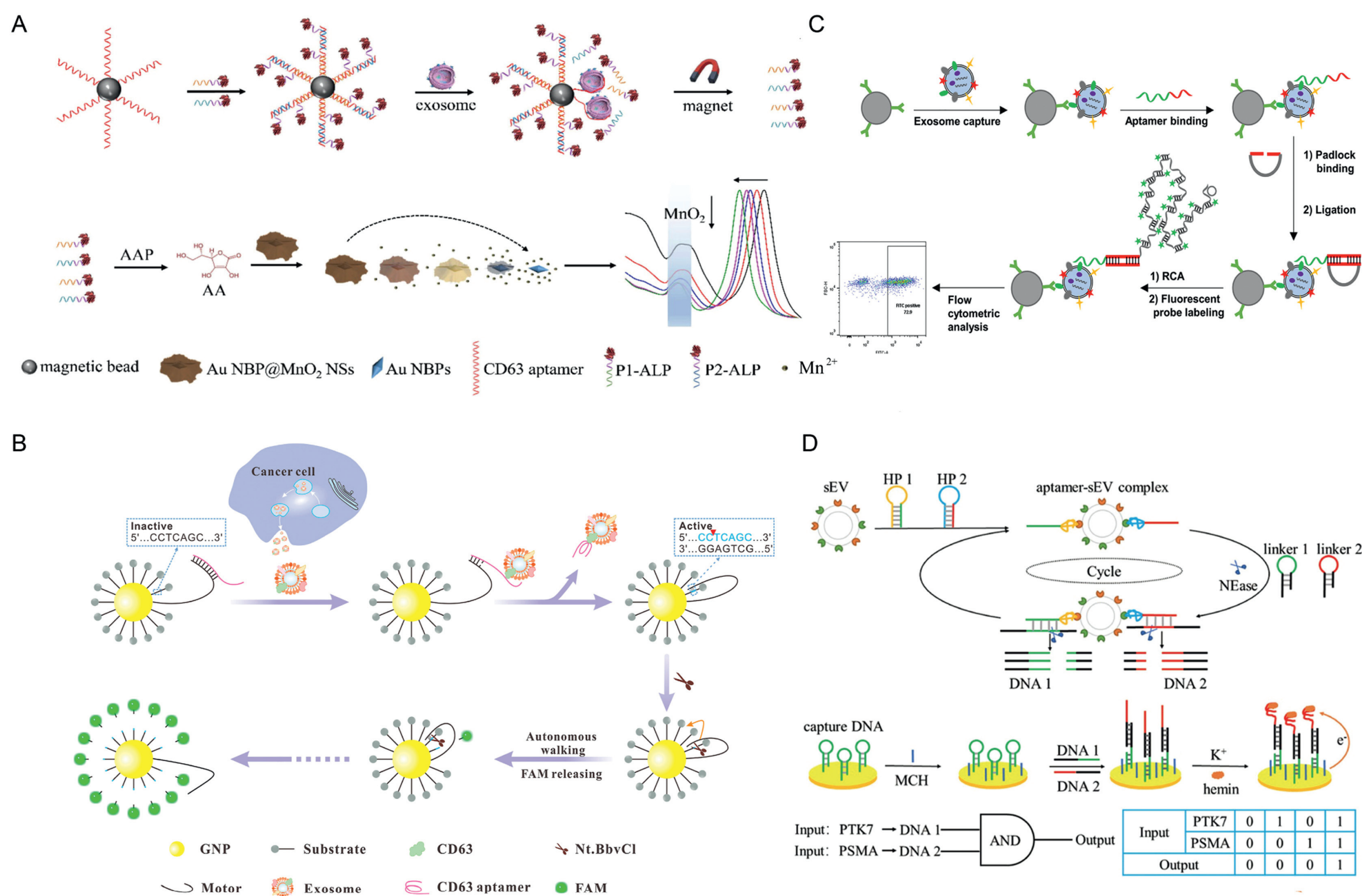

Fig. 3. One-dimensional DNA nanostructures assisted EVs biosensors. (A) Exosome-triggered competitive reaction translating the measurement of exosomes into alkaline phosphatase quantification, and etching of gold nanobipyramid@MnO_2 nanosheet nanostructures for plasmonic signal amplification. Copied with permission [51]. Copyright 2020, American Chemical Society. (B) DNA walker containing gold nanoparticles, fluorescein-labeled strands and CD63 aptamer-locked strands was triggered by EVs, and the movement of DNA walker led to fluorescence recovery. Copied with permission [70]. Copyright, 2020, Elsevier. (C) Magnetic beads based EVs capture and RCA assisted flow cytometry analysis for EVs detection. Copied with permission [82]. Copyright 2021, American Chemical Society. (D) Dual-aptamer-assisted electrochemical detection of EVs. Copied with permission [95]. Copyright 2020, American Chemical Society.

to evaluate multiple EVs surface proteins. Lin and co-workers developed a dual-aptamer activated system for EVs detection [90]. Two types of designed aptamers were used to simultaneously recognize both biomarkers on one EV. Subsequently, the extended end of two aptamers were ligated and droplet digital PCR was performed.

For further improvement in multiplex analysis of EVs surface biomarkers, computing [91,92] and nanomaterials [93,94] are integrated with DNA nanostructures. For example, Yu and co-workers used a dual-aptamer-assisted AND logic gate to analysis two tumor-related proteins (tyrosine kinase-like 7 and prostate-specific membrane antigen) on CCRF-CEM EVs (Fig. 3D) [95]. EVs containing both target protein biomarkers could trigger a target recycling process, which led to the release of the hybridization region of the initial platform. These DNA strands were then detected by a AND logic gate-based electrochemical biosensor.

Although 1D DNA nanostructures play significant important roles in DNA nanostructure-based biosensing, drawbacks exist due to their low dimensionality, which results in less effective space for molecules immobilization and limited shape design.

3.3. Two-dimensional DNA nanostructures

Compared to 1D nanostructures, two-dimensional (2D) nanostructures exhibit several advantages: enhanced enzyme-mimic properties, higher specific surface area which provide much more active sites for molecules immobilization, the ability to be used as template for conjugation with functional hybrid nanomaterials [96]. Thus, 2D DNA nanostructures can be used to construct cascade signal amplification system for EVs detection [97–99]. Gao and co-workers reported a dual signal amplification method based on DNA dendrimers self-assembly [100]. The presence of target EVs triggered hairpin DNA cascade reaction (HDCR). The second signal amplification was achieved by adding DNA dendrimers into the solution to form a hyper-branched structure.

Another strategy to generate 2D nanostructures is using RCA. In the past few years, new developments in the use of RCA have appeared in the area of nanotechnology [101]. RCA now can be tailor-designed to produce structural DNA motifs, or to generate large-scale 2D materials such as DNA origami and nanoribbons. Cao and co-workers conducted hyperbranched RCA (HRCA) on the surface of EVs (Fig. 4A) [102]. The hyperbranched DNA nanostructures lowered the pH of the solution, leading to the disassembly of pH-responsive enzyme-encapsulating metal-organic frameworks (MOFs). The released enzyme then rose the amplified electrochemical responses. Another strategy developed by our group used the branched RCA to load fluorescent dyes to achieve signal amplification [78]. EVs capturing plays an importance role in EVs detection, Ji and co-workers constructed a 2D DNA-RNA hybridized membrane using RCA and rolling circle transcription (RCT) [103]. After dual polymerization, the DNA and RNA replicons were entangled to generate an initial membrane to serve as a collecting platform of EVs.

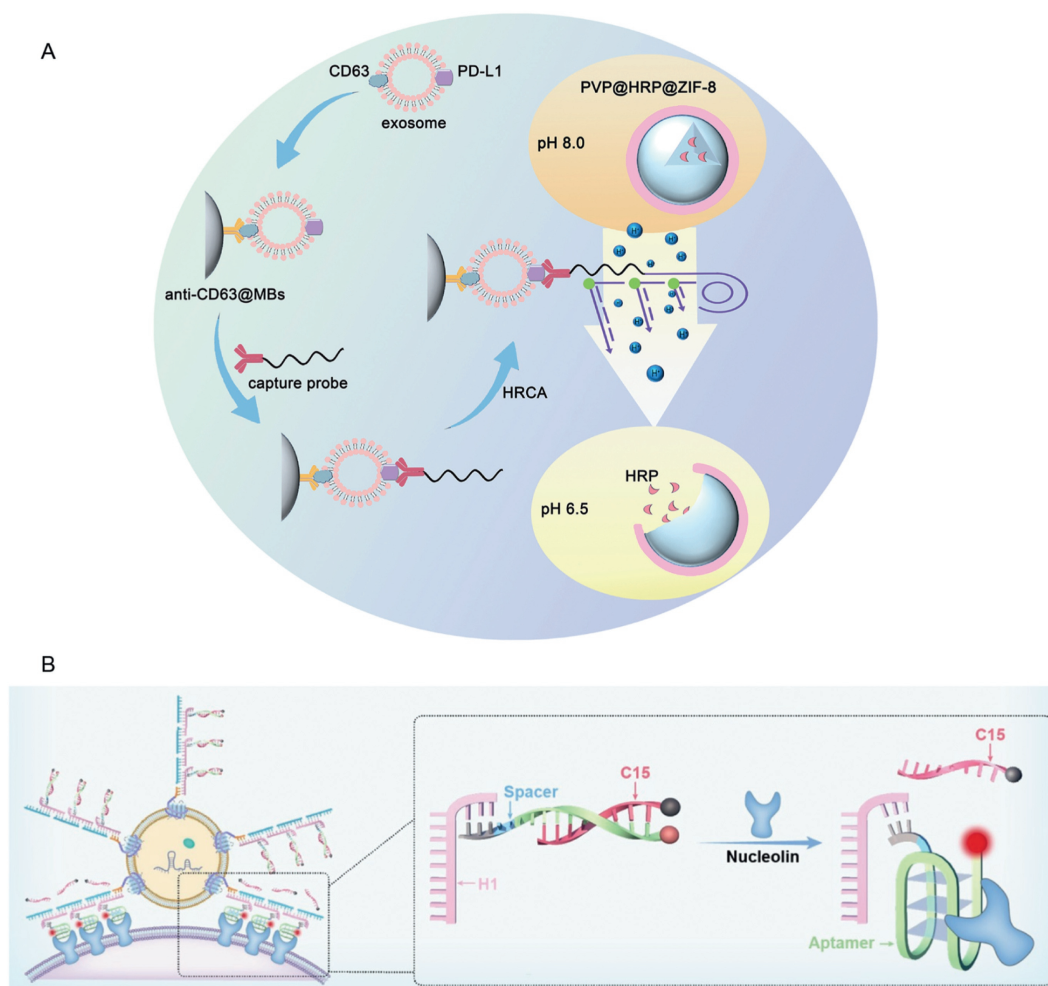

Fig. 4. Two-dimensional DNA nanostructures assisted EVs biosensors. (A) HRCA and MOFs assisted electrochemical biosensor. Copied with permission [102]. Copyright 2020, Elsevier. (B) Polymeric DNA structures produced *via* HCR were engineered onto exosome membranes to serve as sensing probe. Fluorescence recovery occurred upon encountering targeted cells. Copied with permission [104]. Copyright 2022, American Chemical Society.

HCR can also be modified to form 2D nanostructures to assist EVs modification and analysis. Liu and co-workers performed HCR on the surface of EVs to produce engineered EVs (Fig. 4B) [104]. Each monomers hybridization provided biding site for hybridization with aptamer, resulting in multivalent aptamer-engineered EVs. After that, 'off-state' aptamer sensing probes were constructed and the fluorescence signal could only be recovered when the targeted interaction occurred.

DNA tiles have the ability to self-assemble into 2D arrays. Liu and co-workers reported an electrochemical biosensor based on localized DNA cascade displacement reaction (L-DCDR) and DNA nanosheet [105]. They used nine DNA strands to build the nanosheet and used it as a nanocarrier to load electroactive methylene blue (MB). The addition of target exosomal miRNA triggered the L-DCDR and the nanosheet-MB complex was captured on the electrode surface generating the amplified electrochemical signal.

As mentioned above, 2D DNA nanostructure owns obvious advantages compared with 1D nanostructure. On the other hand, the complex construction of 2D nanomaterials requires higher manufacturing cost, more difficult design and preparation.

3.4. Three-dimensional DNA nanostructures

DNA nanotechnology revolutionized the capabilities to fabricate arbitrary three-dimensional (3D) shapes. With versatile structures and high-precise orientations, 3D DNA nanostructures have shown promising applications in biosensing.

One of the flaws in DNA nanotechnology at the beginning was the rigidity of DNA nanostructures due to the unsatisfied mechanical property of a single-DNA duplex. To solve this problem, nanoparticle such as gold nanoparticle [AuNP], which exhibit unique physicochemical property [106] are used to serve as a core to template and organize DNA. Moreover, the combination with nanoparticles adds functionality to the DNA nanostructures [107]. Chen and co-workers synthetized a DNA hydrogel functionalized AuNPs (H-Au) for real-time surface plasmon resonance imaging of cancer-derived EVs [108]. Prostate specific membrane antigen (PSMA), a protein biomarker with diagnostic value expressed on the surface of EVs was served as the target. The presence of EVs could trigger the release of DNA linker from PSMA-specific aptamer-DNA linker complex, which led to the hybridization of capture probe on the sensor chip with H-Au to achieve efficient signal amplification.

DNA lattices assembled from DNA tiles have highly mechanical rigid architecture. Wherein, DNA tetrahedra seems to be the mostly used 3D nanostructures in the field [109,110]. Han and co-workers reported the detection of mRNA in EVs using DNA tetrahedron (Fig. 5A) [110]. Target mRNA enhanced fluorescence resonance energy transfer signal was further amplified by thermophoretic effect to accomplish ultrasensitive detection. Xu and

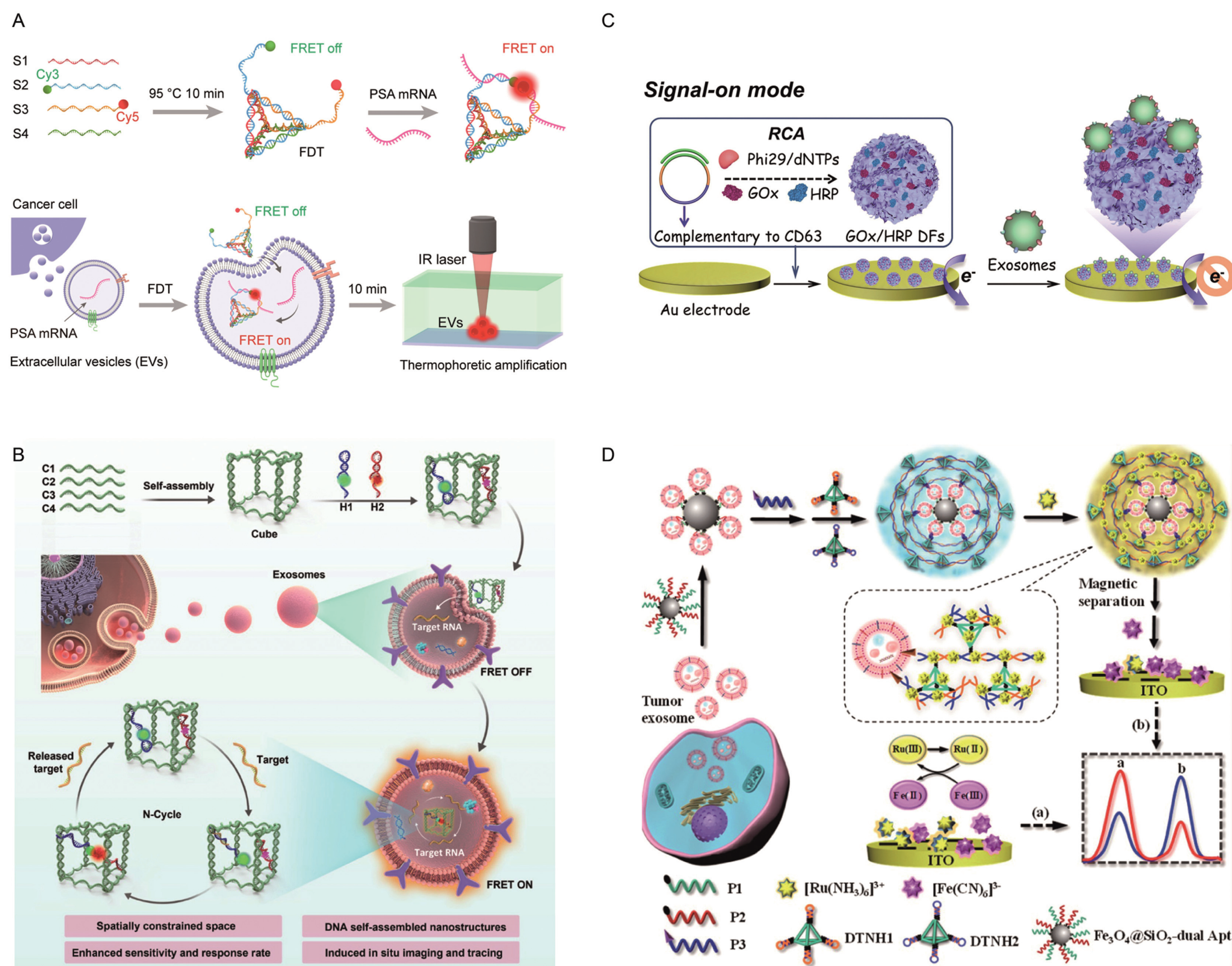

Fig. 5. Two-dimensional DNA nanostructures assisted EVs biosensors. (A) DNA tetrahedron-based thermophoretic assay for the detection of mRNA in EVs. Copied with permission [110]. Copyright 2021, Elsevier. (B) DNA cascade nanostructure for exosomal miRNA detection *in situ*. Fluorescence signal was enhanced *via* target miRNA triggered catalytic cycle. Copied with permission [115]. Copyright 2022, American Chemical Society. (C) RCA based electrochemical biosensor. Copied with permission [120]. Copyright 2021, Elsevier. (D) Target triggered DNA tetrahedron-based hyper HCR to construct DNA superstructure, which then bound with Ru(III) to produce electrochemical signal. Copied with permission [121], Copyright 2021, American Chemical Society.

co-workers used a switchable DNA tetrahedra scaffold for the detection of breast cancer-derived EVs [111]. The tetrahedra contained G-quadruplex mimicking DNAzyme sequence and biomarker specific aptamer sequence. With the presence of target EVs, G-quadruplex was released from the tetrahedra scaffold and catalyzed the colorless substrate into a colored product. DNA tetrahedra can also be used to capture EVs instead of unmodified aptamers to avoid entanglement, aggregation and spatial hindrance effect that influence the recognition efficiency of capture probe. Liao and co-workers used DNA tetrahedra to capture EVs onto the Au film. Then polydopamine-functionalized AuNPs were bound with the captured EVs to assist surface plasmon resonance [SPR] signal amplification [112]. Similarly, Jiang and co-workers anchored DNA tetrahedra on the gold electrode surface to capture EVs [113]. Other DNA lattices have also been built to assist the molecular analysis of EVs [114]. Chen and co-workers synthesized a DNA nanoframe for *in situ* monitoring of exosomal miRNA and exosome tracing (Fig. 5B) [115]. The proposed nanoframe contained a nanocube and two fluorescence labeled hairpins. Target miRNA hybridized with the hairpins to form a duplex structure, leading to fluorescence resonance energy transfer (FRET); and the released miRNA could participate in the new catalytic cycle.

With the assisted of magnesium pyrophosphate, RCA products can be used to construct 3D nanostructures such as hydrogels, nanoflowers and other high-performance scaffolds [116]. Zhang and co-workers reported a localized fluorescent imaging of multiple proteins on individual EV [117]. Aptamers specific to surface biomarkers were bound with EVs to act as primers, followed by RCA to produce localized fluorescent signals. The RCA products were confirmed to undergo structural change from long single-stranded to petal-like structures. As colorimetric biosensors are able to deliver naked-eye and low-cost detection [118], in another example, RCA products were used to encapsulate horseradish peroxidase [HRP] and then bound with EVs to generate colorimetric signals [119]. Similarly, Yan and co-workers used DNA nanoflowers synthesized by RCA to load glucose oxidase/HRP (Fig. 5C) [120]. Ultralong-chain RCA products self-assembled into porous DNA-inorganic nanoflower was loaded with glucose oxidase [GOx] and HRP. The HRP-NF nanostructure then served as recognition and signal-generating elements of the electrochemical biosensor.

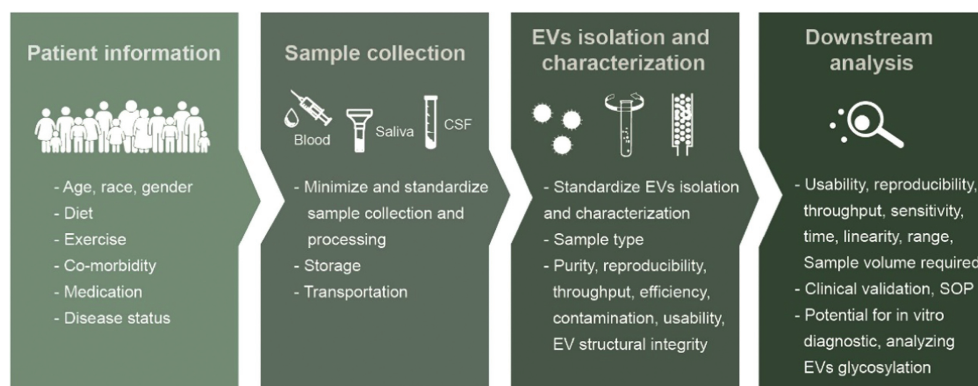

Fig. 6. Evaluation of DNA nanotechnology assisted EVs detection and diagnosis assays for potential use in clinical practice.

Moreover, the approaches mentioned above can be mixed very well. Yang and co-workers combined DNA tetrahedra with HCR for the quantification of EVs (Fig. 5D) [121]. Target EVs were first captured using magnetic beads. HCR was triggered by the probes anchored on the EVs membrane and DNA tetrahedra was added into the solution to form a DNA superstructure. The 3D nanostructure could bind a large amount of $Ru[NH_3]_6{}^{3+}$, leading to an obvious signal change. Wu and co-workers developed a ratiometric DNA machine by the combination of DNA walker and cascade amplification to analyze urinary EVs [122]. The DNA machine contained a padlock probe, anchor strands and nucleic-acid-stabilized silver nanoclusters. The presence of EVs activated DNA machine and produced a ratiometric fluorescence signal.

Although 3D DNA nanostructures show great potential in EVs biosensing, it is premature for their practical applications. There are still some limitations including relatively high manufacturing cost, complex sequence design and optimization, time-consuming preparation, need for exact stoichiometric and purity control.

4. Discussion

4.1. Attributes of DNA nanotechnology-assisted EVs analysis

Nowadays, EVs are studied extensively to explore their diagnostic potential for a number of diseases with practical clinical application. However, the small size, heterogeneity, relatively low abundance of surface proteins and complex pre-analytical steps pose great obstacles in EVs detection and diagnosis. From the summary above it can righty be rightly stated that the filed of DNA nanotechnology has great potential to be used in development of novel detection platforms of EVs. The unique characteristics of DNA nanostructure have been widely exploited in development of platforms for EVs detection. In surface-based methods, aptamers are widely used as recognition element, wherein entanglement and aggregation of aptamers at the biosensing interface hinder the efficiency of target capture. DNA nanostructures such as DNA tetrahedra [112] and DNA walker [68], can either reduce spatial hindrance effect, enhance target accessibility, or allow dynamic target capturing and release to provide more binding sites. The programmability of DNA nanostructure provides novel and practical solutions for EVs detection based on structural change of DNA in response to target EVs. DNA walker, DNA logic gates, DNA-assisted target recycling process and other strategies have been established [65,69,70]. Besides the efficient control of their structure, DNA nanostructures can be easily modified with chemical molecules and functional materials making them suitable for development of versatile biosensors. For example, nanoflowers was prepared for enzyme loading [119], long tandem DNA was produced for signal amplification [78], and G-quadruplex was designed to serve as mimic enzyme [80]. Furthermore, the excellent biocompatibility of DNA nanostructure offers opportunity of EVs detection in living biological systems [104,115].

Despite the advantages mentioned above, DNA nanotechnology assisted EVs analysis is still at the stage of exploration and there remains several challenges. For example, nanostructures constructed by the tile-based bottom-up assembly requires computer-aided programs. Precise stoichiometric and purity control is required. Nanostructures assembled by origami are relatively expensive and require time depleting steps to purify and accumulate to meet the demands of practical application. Furthermore, the thermal stability and resistance to nucleases degradation of DNA nanostructures is concerning.

4.2. Challenges and perspectives

Before DNA nanotechnology assisted EVs detection and diagnosis can be translated into clinical practice, there remains several obstacles. The main issue is lack of EVs isolation and analysis methods with high reproducibility which can be suitable for clinical use. Some of the reported EVs detecting methods demonstrated the proof of concept but may not be feasible for large-scale clinical applications as rigorous validation were not performed. Besides stability and reproducibility, the detection throughput also requires improvement for practical applications. Microfluidic devices with enhanced accuracy and stability [123,124] are suitable in this situation. Furthermore, sample collection, storage and transportation prior to downstream EVs analysis should be standardized, and detailed information about donor characteristics should be taken into consideration [15]. To estimate the performance of EVs detecting methods, clinical validation should be conducted in both retrospective cohort and prospective cohorts [125–127].

In the future, more efforts should be paid to solve the above problems. Furthermore, identification of the associations between EVs and diseases using experiments is burdensome, computational methods can be employed to complement the experiments [128,129]. Researchers are also encouraged to establish detection assays that integrate EVs isolation and analysis directly from body fluids as these methods are more clinically feasible. In addition, the current EVs detection and diagnosis studies mainly focus on EVs nucleic acid and protein cargoes, and glycan-based biomarkers are neglected. As glycosylation is closely related to multiple pathophysiological process including cancer progression and metastasis [130,131], specific analysis of EV glycosylation is benefit to distinguish between different subpopulations of EVs with overlapping size and biochemical characteristics. Hence, further research focusing on analyzing EVs glycosylation is required. We suggest a checklist of items to consider in clinical translation of the DNA nanotechnology assisted EVs biosensors (Fig. 6).

5. Conclusion

EVs have the potential to be ideal biomarkers to improve diagnosis and prognostic evaluation for multiple human disorders. DNA nanostructures exhibit unique properties due to their programmable and reconfigurable structure, intrinsic biocompatibility and biodegradability, easy to modify with various chemical groups and nanomaterials. DNA nanotechnology assisted EVs detection and diagnosis offers high sensitivity and selectivity, enhanced target capture efficiency and the ability to develop versatile platforms. On the other hand, challenges stemming from the unstandardized operation procedure prior to downstream EVs analysis, lacking of rigorous validation and inherent shortcoming of DNA nanotechnology have impeded the progress of translating laboratory research into clinical practice. Efforts need to be taken to establish standardized validation procedures assessing EVs assays and to reduce the production cost of DNA nanostructure. High-throughput methods and analysis of EV glycosylation are also encouraged. And as artificial intelligence (AI) has drawn great attention in the last few years, bioinformatics tools can be valuable for EVs studies. Although commercial viability is not a reality yet, and only a few of the reported methods performed comparison tests to commercially available chip [117], it is believed that DNA nanotechnology will open up new possibilities in the area of EVs detection and diagnosis.

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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