Electrochemical Detection of Cancer Fingerprint: A Systematic Review on Recent Progress in Extracellular Vesicle Research from Lab to Market

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Abstract

Cancer is a major global health burden and accounts for the second-highest mortality rate around the world. Despite significant advances in cancer research, minimal/noninvasive diagnostic strategies for early detection remain elusive, leading to delays in therapeutic intervention and affecting the overall survival chances. Most cancers exhibit no specific symptoms in the early stages. However, upon metastasis to nearby organs, certain symptoms become prominent and detectable. Conventional tissue biopsy performed in these cancers is not only invasive but is also limited in its diagnostic efficacy by the presence of tumor heterogeneity. In this regard, liquid biopsy has brought a revolution in early cancer diagnosis. Extracellular vesicles are among some of the reliable biomarkers for liquid biopsy. Recent advancements in technology have laid a strong foundation in understanding the role of extracellular vesicles in cancer progression. In this review, we briefly present the multifarious roles of extracellular vesicles toward an accurate cancer diagnosis. Moreover, the recent progress in extracellular vesicle liquid biopsy biosensors is also discussed, with a focus on electrochemical biosensors. Lastly, the requisite measures to be taken to improve the performance of the existing biosensors, with an aim of bringing them to the commercial point-of-care (POC) market, are concisely summarized.

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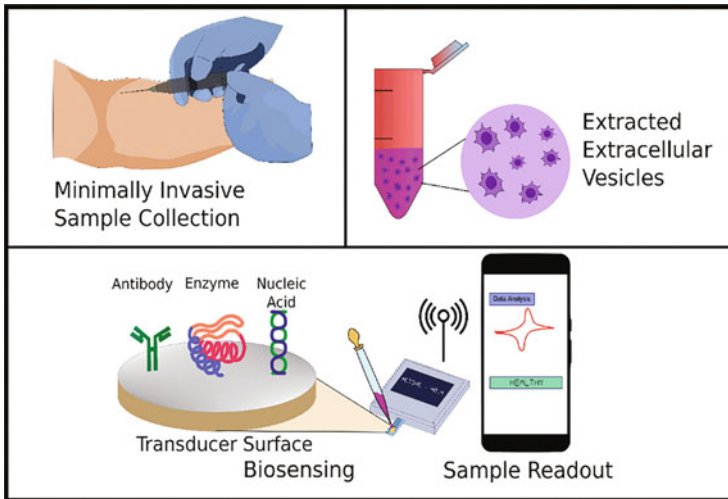
1   Introduction

Cancer is the second major cause of death worldwide [1]. Tumor heterogeneity,
chemotherapeutic resistance, and delay in accurate diagnosis are some principal
challenges associated with cancer [2]. Though the conventional tissue biopsy-
based cancer diagnosis has advanced significantly, high turnaround time, the inva-
sive procedure makes it very difficult for routine cancer diagnosis. Due to those
technical and biological complications with tissue scientists are giving more empha-
sis to liquid biopsy for many years. The advancement of liquid biopsy opens an
excellent outlook for future clinical diagnosis. In comparison with tissue biopsy,
liquid biopsies are less risky and can capture the complete tumor heterogeneity of a
patient [3]. In the case of liquid biopsy, the proteogenomic alternation of cancer can
be detected from tumor-shredded components in different human bioliquids such as
blood and urine [3]. Recently, extracellular vesicles (EV) have appeared as an
excellent alternative biomarker for liquid biopsy. Howbeit, earlier EVs were consid-
ered cellular debris, but are currently acknowledged as one of the main factors in
intercellular communication [4]. EV-incorporated biomacromolecules play a key
role in mimicking the pathophysiological microenvironment of the parent cell
[4]. Thusly, EVs can modulate cancer development and offers great promise as a

diagnostic element. Despite the diagnostic advances, the lack of standardization in EV separation and detection brings a great challenge in actual clinical translation.

Various conventional techniques such as ELISA, blotting, microarray, and RT-PCR are generally used to characterize cell-derived EVs [5]. Nevertheless, such conventional techniques require a substantial amount of sample which is unsuitable for repeated clinical diagnosis. Moreover, the requirement of complex instrumentation, high turnaround time, and sample pre-treatment with conventional techniques limit the utility of EVs in clinical settings. Electrochemical biosensors are a type of bioanalytical device that can convert biochemical interactions into a measurable electrical signal. These biosensors contain solid electrode surfaces to immobilize the biorecognition elements like antibodies, proteins, aptamer, and enzymes, and upon specific biochemical interactions with a target of interest electrical signals are generated which are measured in terms of the analyte concentration [6]. These electrochemistry-based biosensing platforms can be immobilized easily for onsite target detection. Considering the recent progress and difficulties in EV research with conventional techniques, electrochemical nano biosensor shows enhanced performance and accurate detection. An accurate point-of-care (POC) medical device can solve these purposes. An ideal POC device must meet all world health organization (WHO) recommended ASSURED criteria (affordable, sensitive, specific, user friendly, rapid and robust, equipment-free, and deliverable) [7].

Herein, we present the biogenesis and role of EV in cancer progression. We also discuss the role of EV as a promising biomarker in liquid biopsy. We highlight a comprehensive review of different electrochemical signal detection platforms and focus on the advantages of conventional EV sensing techniques. Further, we highlight the different applications of electrochemical biosensors in cancer-specific EV detection. Finally, we discuss the direction of future research that promises to ravel a new horizon in cancer diagnosis with electrochemical liquid biopsy sensors. We believe that with the progress of science and technology and newborn applications in different electrochemical biosensors, it is possible to facilitate personalized diagnosis in near future.

2   Early Detection Is the Best Answer for Cancer

Early diagnosis of cancer is of paramount importance for enabling successful treatment. The complex eukaryotic cellular machinery regulates cellular function in the human body. Aberrations in molecules associated with signal transduction often underlie the initiation and progression of cancer. If any of these signals get dysregulated, healthy cells might start to multiply rapidly to form a tumor [8]. Uncontrolled division of cancer cells can invade nearby tissues to form a new tumor. According to reports over 19.3 million cases of cancer are diagnosed in 2020, worldwide. In line with the Indian cancer registry program reported over 1,392,179 cancer cases in India for the year 2020 [9]. Cancers are often exhibited no clinical symptoms and hence treatment is overlooked. For that reason, most of the

patients are negligent and ignore proper diagnosis processes. Besides, healthcare literacy is very low in low-middle income countries like India. Furthermore, complex diagnosis processes and associated costs increase the anxiety level of patients. Early detection of cancer can decrease morbidity and can increase the survival rate. The majority of cancer-related deaths comes from metastasizing malignant cell [8]. Once a malignant cell starts metastasizing, then it is only treatable not curable. The delay in the specific and timely detection limits the access to treat cancer. Before becoming symptomatic, if it is detected then the tumor can be surgically removed or systematic treatment can be prescribed. Reports indicated that in 53% of cases when lung cancer patients are diagnosed at stage I, patients survive more than 5 years [10]. The extent of systematic treatment depends upon the metastasized potential and tumor size at the time of detection [8].

Although scientists put their effort to recognize early cancer symptoms but most of the time those symptoms are highly non-specific. Hence, to avoid conventional tissue biopsy complications, and to capture the whole proteogenomic profile for particular cancer, it is very important to recognize cancer-associated biological markers with minimally invasive procedures [3]. Clinical diagnosis of cancer with a minimally invasive procedure is termed a liquid biopsy [3]. Liquid biopsy studies mainly focus on circulating DNA (ct DNA), circulating RNA (ct RNA), circulating tumor cell (CTC), and extracellular vesicles (EV) that are released by tumor cells in different biological fluids like blood, urine, saliva, and cerebrospinal fluid [11]. The elevated level of these biomarkers might contribute as a predictive signature for the early detection of cancer (Fig. 1).

3 EV, a Promising Youngster in Liquid Biopsy

Recent progress in cancer research has immensely advanced early cancer diagnosis with the development of new and sophisticated approaches. Currently, tissue biopsy is regarded as the gold standard for cancer diagnosis [4]. During tissue biopsy, doctors collect a small part of tissue to examine if it is cancerous. Though tissue biopsy access doctors to determine cancer's grade and give clues to a specific treatment strategy, nevertheless this surgical strategy is invasive, and sometimes tumor organ is inaccessible for biopsy [13]. In addition, a repeat biopsy is not possible for continuous monitoring. Furthermore, high tumor heterogeneity presents a serious challenge to understanding the complete molecular profile of particular cancer by analyzing a small section of cancerous tissue [13]. In addition to that, high turnaround time, high cost, post-clinical complications, and invasive surgical procedure imposes substantial inadequacy on solid (tissue) biopsy [13]. In view of these shortcomings, researchers are increasingly shifting their research focus from solid biopsy to liquid biopsy. Liquid biopsy represents noninvasive diagnostic techniques from non-solid tumor tissue, mostly from easily isolated bioliquids [3]. As the primary tumor grows it releases certain biomarkers into the circulation that can be collected from human bioliquids like blood, urine, saliva, and cerebrospinal fluid. Liquid biopsy biomarkers generally exhibit dynamic changes associated with
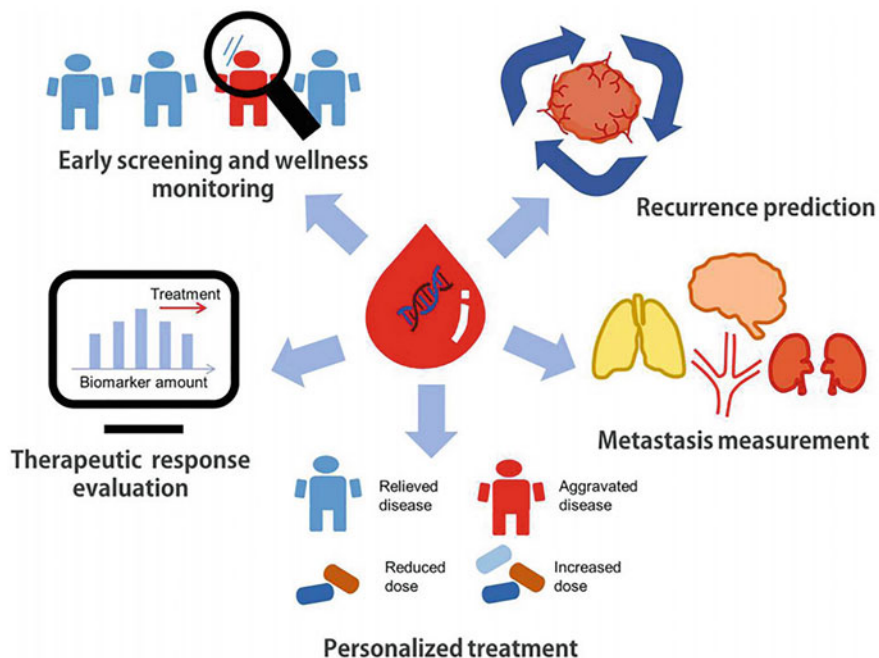
Fig. 1 Liquid biopsy explores different biological markers present in human bioliquids. Liquid biopsy could be an excellent alternative tissue biopsy to analyze the metastatic potential of tumors at a very early stage to predict the diagnosis and prognosis of cancer. Reprinted from [12] Copyright 2021, with permission from Elsevier

cancer, emerging as a promising diagnosing tool for both early and metastatic cancer (Fig. 2).

In light of that, as the genetic makeup of cancer cells evolves, liquid biopsy is very significant for repeated monitoring [13]. Based on bioliquid-derived components, liquid biopsy biomarkers can be divided into circulating tumor DNA (ctDNA), circulating tumor RNA (ctRNA), circulating tumor cell (CTC), and extracellular vesicles (EV) [11]. Recent studies indicate that malignant cells secreted EVs convey oncogenic factors between healthy cells. The intake of cancer cell-derived EVs may induce a malignant transformation in recipient cells [4]. Among other EV-incorporated cargoes oncogenic proteins and tumor-suppressive micro RNAs (miRNA) present superior diagnostic potential in early cancer diagnosis.

EVs extends substantial advantages over the other liquid biopsy biomarkers in various aspect. Lipid double-layer contained tumor-specific cargoes are free from enzymatic degradation in the case of EVs. EVs often manifest pathological stages of particular cancer by expressing certain surface proteins that can be easily used for isolation of EV subpopulation and disease monitoring. By and large, in terms of the incorporation of biological information and diagnosis sensitivity, EVs offer higher accuracy than others [4]. The concept of early cancer detection with EVs is still
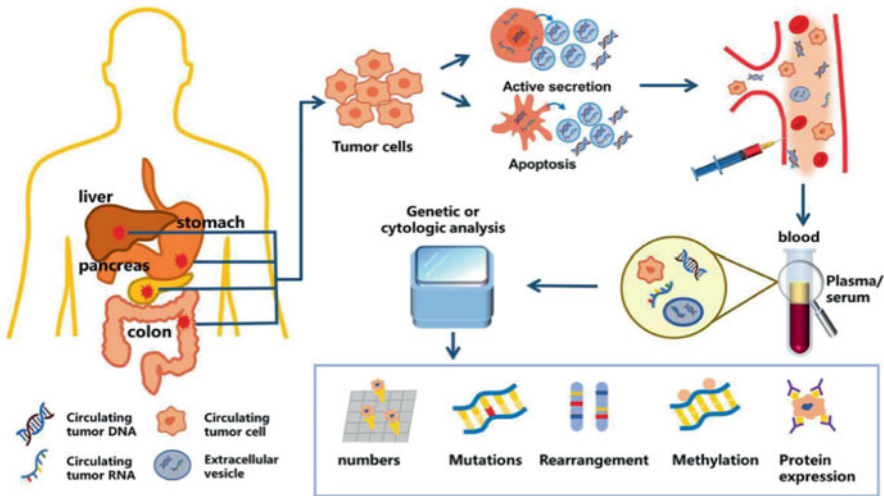
Fig. 2 Verities of biomarkers present in the circulation—circulating tumor cells, circulating tumor nucleic acids, tumor platelets, and extracellular vesicles are detected in liquid biopsy. Dysregulated proteogenomic alternations associated with those biomarkers can be used in early cancer diagnosis. Reprinted from [12] Copyright 2021, with permission from Elsevier

young and numerous clinical studies are in process to convene the complete cancer diagnostic potential of EVs.

4   Conventional EV Detection Techniques

For diagnosis, the isolated EV has to be pure. EV isolation techniques are generally categorized based on charge, size, and immunoaffinity. Currently, ultracentrifugation, immunoaffinity chromatography, and size exclusion chromatography are considered the gold standard for EV isolation [14] (Fig. 3). However, most of the current methods are labor and time-intensive process. On top of that, isolated vesicle quality is far from pure [5]. Scientists generally perform a combination of techniques to attain high purity. Furthermore, the biomechanical properties of EVs highly depend on the isolation technique. Thence, pre-analytical variables are considered to select a suitable technique [14]. Besides, conventional techniques, many new approaches are emerging. For instance, dielectrophoresis, field flow fractionation, hydrophobic interaction chromatography, field-free viscoelastic flow, etc. [5]. MISEV has listed different EV isolation, characterization, and detection techniques to recommend as per the need of the research. With the advancement of new techniques in this particular field, MISEV has revised its recommendation over the years. The standardization of an accurate EV characterization and detection technique is so compelling to understanding the physicochemical properties of an EV subpopulation [5].
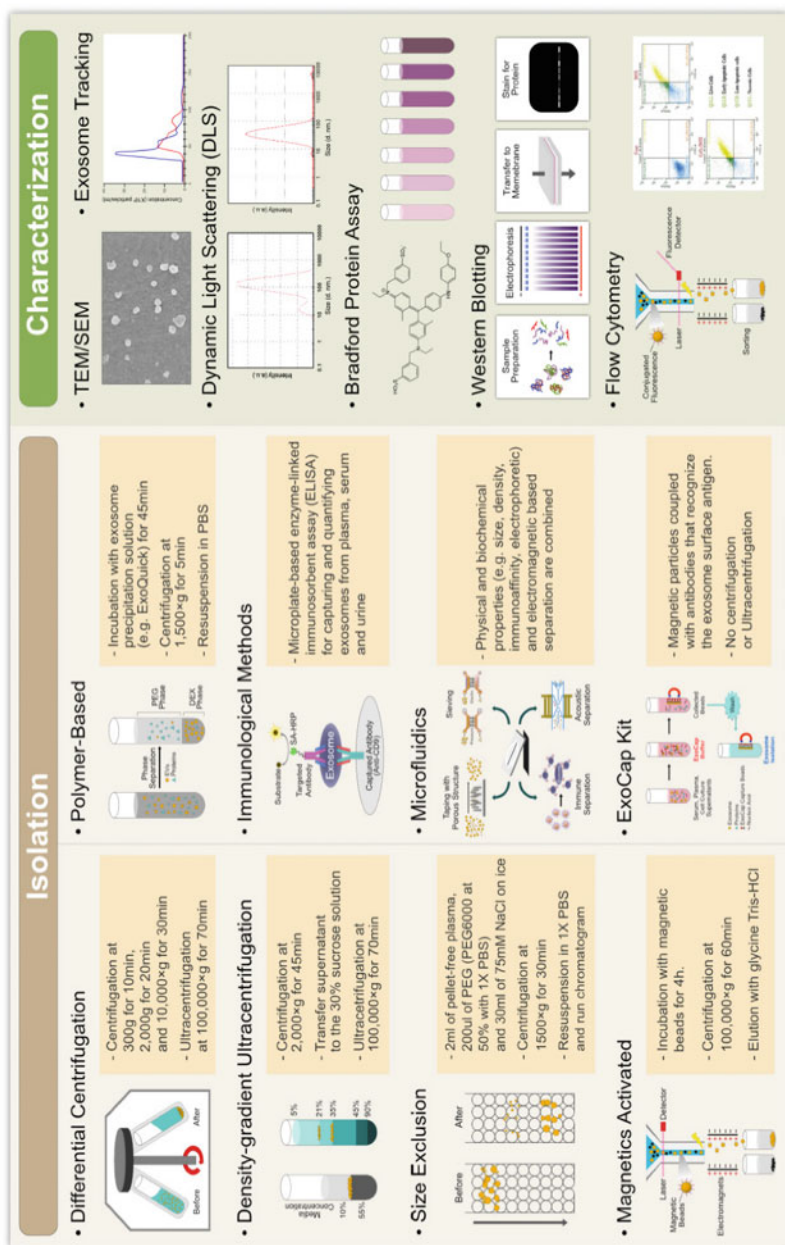
Fig. 3 The conventional techniques to isolate and characterize extracellular vesicles. Reproduced from the open-access article [14] under the terms of Creative Commons CC BY

Table 1 Conventional techniques for EV characterization

S. no.	EV characterization techniques	Advantages	Disadvantages	
1	Atomic Force Microscopy (AFM)	• Highly sensitive • Works in physiological condition • High Resolution	• Background noise can hamper reproducibility	[15]
2	Dynamic light scattering (DLS)	• Independent of sample turbidity • Simple experimental set-up • Accurate	• Highly sensitive toward mechanical noise	[16]
3	Flow cytometry	• High throughput absolute counting • Accurate • Low cost	• Expensive • Difficult to maintain physiological environment	[17]
4	Nanoparticle tracking assay (NTA)	• Accurate • Reliable	• Parameter optimization is very significant	[18]
5	Surface plasmon resonance (SPR)	• Sensitive • Label-free • No need for statistical calibration	• Instrument size is very big • Low selectivity	[19]
6	Microfluidics	• Small sample volume is required • Rapid • Low cost	• Adsorption of air bubbles in small channels • Lack of high reproducibility	[20]
7	ELISA	• Rapid • Highly specific	• The enzyme might lose the functional activity • Low sensitivity • High cost	[21–23]
8	Western blot	• A widely accepted standardized protocol is available • EVs can be visualized directly	• Complex • An experienced technician is required • Expensive	[24, 25]
9	RT-PCR	• High sensitivity • The detection range is high • No post PCR process is required	• Technically demanding • Expensive • Time consuming	[26–28]

For the quantitative and qualitative validation of EV-associated biomarkers variety of techniques are generally performed (Table 1). Most common physical methods such as nanoparticle tracking analysis (NTA), Microscopy (EM, AFM) are used to characterize the extracellular vesicles, whereas Western Blot, ELISA, and RT-PCR are used to determine the protein or nucleic acid content of EVs.
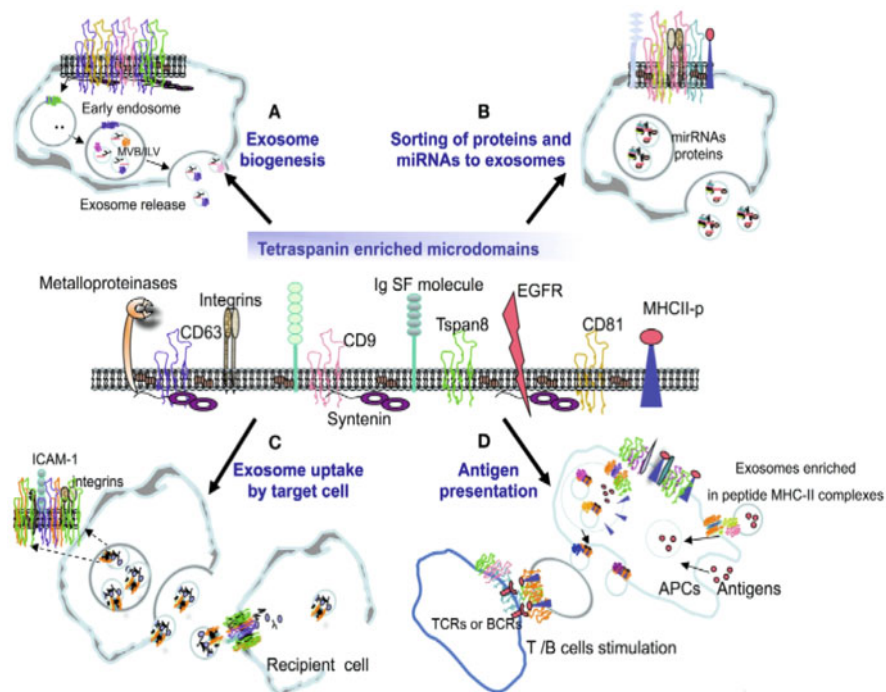
Fig. 4 Tetraspanin proteins abundantly cover the EV membrane surface. These highly glycosylated transmembrane proteins are associated with different biological processes like EV biogenesis, protein sorting inside the vesicle, and immunogenic response. Besides, conserved residues of tetraspanins signify characteristic features of the EV subpopulation. Reproduced from open-access article [29] under the terms of Creative Commons CC BY

EV-incorporated cargoes are a very important and promising tool from a diagnostic and prognostic point of view [4]. The most regularly used biochemical methods include immune assay, blotting assay, and PCR analytic methods to determine the protein or nucleic acid content of EV.

Currently, various conventional methods are used for the isolation and characterization of EVs (Fig. 4). Apart from the above-mentioned methods, different molecular detection strategies such as Raman spectroscopy, FTIR, surface plasmon resonance, and circular dichroism are regularly used for EV analysis [30–32]. Despite that fact, there is no standardized method is available now for EV detection and characterization. The application of EV research is very crucial in early disease diagnosis. Unfortunately, most of the conventional strategies demand a benchtop set up for diagnosis. Furthermore, a large amount of bioliquid is required which is not suitable for repeated analysis. In a situation like this, advancement in

point-of-care diagnosis is preferable. In the following section, we will explore the insights of the electrochemical biosensor concerning point-of-care EV research.

5   Electrochemical Detection of EVs

EV membrane-enriched tetraspanins constitute a major class of transmembrane proteins that enrich the EV membrane surface by forming a cluster of "transmembrane enriched microdomains" [29]. Out of the five critical microdomains, a highly variable extracellular loop is mainly involved in EV-mediated intercellular communication (Fig. 4).

Due to the capacity of highly specific biochemical interactions, the extracellular domain is functionally very crucial [33]. Not only, EV surface proteins but the nucleic acid composition inside EV was found to alter the biological activities of cells that take them up. A detailed multi-omic study suggested the presence of a high concentration of different micro RNAs (miRNA) in EV [34]. The double membrane boundary of EV provides stability to incorporated RNAs from cytoplasmic nucleases [4]. Not only miRNAs but noncoding (nc) RNA, genomic, and mitochondrial DNAs are also transported from one cell to another through EV [4]. The computational correlation of EV-incorporated nucleic acid cargoes displays the pathophysiological insights of secreted cells. Boriachek et al. developed a label-free, amplification-free electrochemical biosensor for the detection of EV-incorporated miR-21 from breast cancer cell lines [35] (Fig. 5).

With a very simple yet accurate detection platform, they succeed to achieve a LOD of 1 pM with a dynamic range of 0.2–20 pM [35]. Such nucleic acid base sequence information in EV exhibited the differential expression pattern between healthy controls and cancer patients, promising the potential to be used in early diagnosis.

The large repertoire of antibodies (immunoglobins) with different antigen-binding specificity makes them an excellent biorecognition molecule. Kilic et al. designed a label-free electrochemical biosensor to recognize vesicle-expressed CD81 tetraspanins (Fig. 6). They functionalized biotin-labeled CD81 antibodies on the gold-screen printed electrode with streptavidin-biotin conjugation chemistry [36]. Based on the modified sensor surface and vesicle tetraspanin interaction, with increasing sample concentration, there was a formation of a kinetic barrier for the redox probe. The measurement of charge transfer resistance exhibited a linear range with an accurate detection limit of 77 copies/mL [36].

However, a low concentration of EVs in biological fluid limits its use in clinical early diagnosis. To overcome such difficulties different types of signal amplification strategies have been advanced. Signal amplification techniques are mainly optimized to remove the background signal and amplify the lower limit of biomarker detection. Signal amplification strategies are employed in biosensing to facilitate a proportionate or ratiometric increase in the detection signal generation in response to an analyte recognition reaction. In electrochemical sensors, ultrasensitive detection of the analyte is brought forth by coupling the recognition reaction with a chemical,
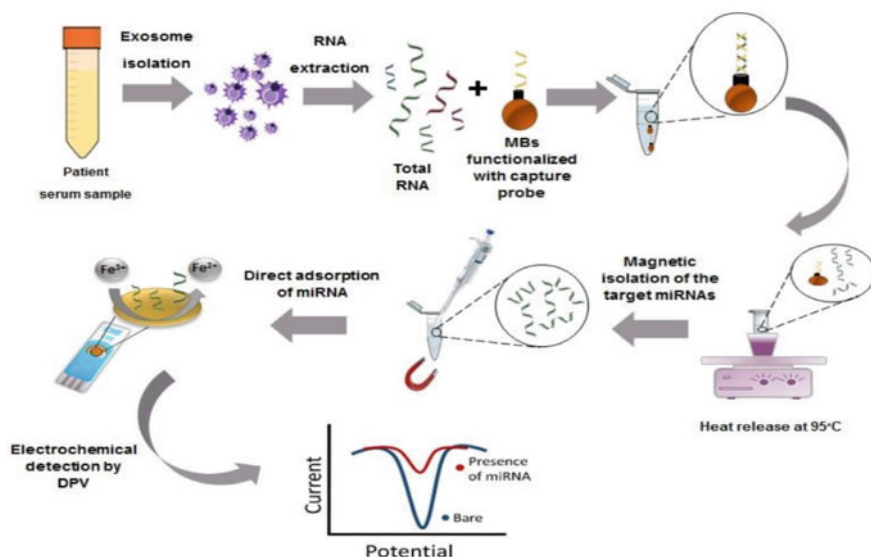
Fig. 5 Schematic representation of steps for the development of an amplification-free electrochemical biosensor to detect EV-incorporated miR-21. Reproduced from [35] with permission from the Royal Society of Chemistry

catalytic or electrocatalytic reaction and rapid electron transfer [37] (Table 2). For EV detection, surface proteins or nucleic acids (such as miRNAs) may be targeted for recognition, and the amplification can be carried out with DNA-based, enzyme-based, or enzyme-free strategies.

5.1   Enzyme-Based Amplification

Enzymes are biological catalysts that enhance the rate of reaction of a substrate to a product. The product formed can be further utilized in an electrocatalytic reaction to produce a detectable electrical signal, or the product can give a direct readout (e.g., colorimetric product formation) [66]. By maintaining optimal conditions for enzyme activity, the reaction rate, and subsequently the detectable signal can be greatly enhanced or amplified. However, the use of enzymes is limited by factors such as surface stability and optimal pH and temperature requirements [67].

Enzyme-mediated amplification for biosensing is a well-established approach, and there have been several reports on enzyme-based biosensors for EV detection. For instance, an enzyme-based multiplexed microfluidic immunosensor was reported by Vaidyanathan and the group for the specific capture of human epidermal growth factor receptor 2 (HER2) and prostate-specific antigen (PSA), expressed on the surface of EVs secreted by breast cancer cell lines [39]. After capturing the protein biomarkers in a sandwich immunocomplex, the signal was generated by the
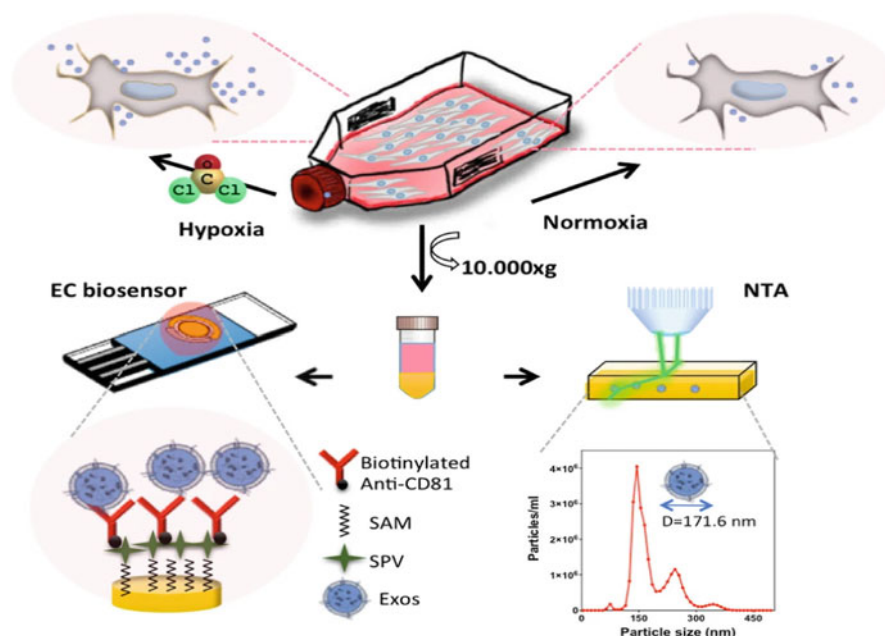
Fig. 6 Indicates the experimental steps of breast cancer cell-derived EV detection. EVs were isolated gold standard ultracentrifugation technique and followed by characterization using NTA. For the electrochemical detection, screen-printed gold electrodes were fabricated with 11 mercaptoundodecanoic acid and neutravidin to capture biotinylated CD81 antibodies. The electrochemical signal of the redox probe was decreased in presence of CD81-positive EVs. The change in the signal was correlated with EV concentration. Reproduced from the open-access article [36] under the terms of Creative Commons CC BY

signal antibody-conjugated horseradish peroxidase (HRP) enzymes with the catalytic oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB), giving a colorimetric readout [39].

5.2 Hybridization-Based Amplification

The complementary base pairing property of nucleic acids to form intricate DNA/RNA structures, as well as replication-based amplification by polymerases, form the basis of amplification strategies in hybridization-based detection platforms [68]. Such structures have found application in many signal amplification strategies in biosensing devices.

Rolling Circle Amplification

Rolling cycle amplification (RCA) is one such technique. RCA is an isothermal amplification technique that uses a ligation primer against a linear DNA sequence, such that the ligation primer terminates on either side in regions complementary to

Table 2 Electrochemical detection of cancer cell-derived EVs

S. no.	Cancer type	EV target	Receptor	Detection platform	Signal label	Detection range	Detection limit	
1	Breast cancer	CD9	αCD9 antibody	Triple pulse amperometry	Horseradish peroxide	2×10^2–1×10^6 copies/µL	1×10^3 copies/µL	[38]
2	Breast cancer	CD81	Cd81 antibody	EIS DPV	$K_3[Fe(CN)_6]^{3-/4-}$	10^2–10^9 copies/mL	77 copies/mL	[36]
3	Breast cancer	HER2, PSA	HER2 antibody, PSA antibody	Tunable alternating current electrodynamic	HRP	4.15×10^4–2.76×10^3 copies/µL	2760 copies/µL	[39]
4	Breast cancer	CD9, HER2	CD9 antibody, HER2 antibody	DPV	$K_3[Fe(CN)_6]^{3-/4-}$		4.7×10^5 copies/µL	[40]
5	Lung cancer	Multiplex detection	CD9, CD63, HER2, PDL-1, IGF1R antibody	Change in current				
6	Kidney cancer	CD81, Syntenin	CD81, Syntenin antibody	EIS	$K_3[Fe(CN)_6]^{3-/4-}$	50 pM – 200 nM	1.9×10^5 copies/mL	[41]
7	Uterus cancer	CD63	PALP antibody, CD63 antibody	Current density	Au-NPFe2O3nanocrystal	10^3–10^7 copies/mL	10^3 copies/mL	[42]
8		EpCAM	EpCAM antibody	CV	Alkaline phosphatase	10–10^6 copies/µL	5 copies/µL	[43]
9	Ovarian cancer	CD63	CD63 antibody	Amperometry	HRP	10^4–10^8 copies/mL	3×10^4 copies/mL	[44]
10	Colon cancer	CD9, CD63, CD81	Antibody	Amperometry	HRP	10^4–10^9 copies/mL	10^4 copies/mL	[45]
11	Prostate cancer	EpCAM	EpCAM antibody	LSV	CuNP		50 copies/mL	[46]

(continued)

Table 2 (continued)

S. no.	Cancer type	EV target	Receptor	Detection platform	Signal label	Detection range	Detection limit	
12	Liver cancer	CD63	CD63 aptamer	SWV	Methylene blue tagged probe	10^6–10^8 copies/mL	10^6 copies/mL	[47]
13	Cervical cancer	PTK	PTK aptamer	DPV	$Ru(NH3)_6^{3+}$		6.607×10^5 copies/mL	[48]
14	Breast cancer	EpCAM	EpCAM aptamer	Amperometry	HRP	5×10^2–1×10^5 copies/µL	285 copies/µL	[49]
15	Breast cancer	CD63	CD63 aptamer	amperometry	HRP	1.12×10^2–1.12×10^8 copies/µL	96 copies/µL	[50]
16	Liver cancer	CD63	CD63 aptamer	DPV	Hemin	7.61×10^4–7.61×10^8 copies/mL	4.39×10^3 copies/mL	[51]
17	Liver cancer	HePG2	HePG2-specific nanotetrahedron aptamer	SWV	$Fe[(CN)6]^{3-/4-}$	10^5–10^{12} copies/mL	2.09×10^4 copies/mL	[52]
18	Gastric cancer	CD63	CD63 aptamer	CV, DPV	HRP mimicking DNA zyme	4.8×10^3–4.8×10^6 copies/mL	9.54×10^2 copies/mL	[53]
19	Breast cancer	CD63, EpCAM	CD63 aptamer	EIS,DPV,CV	Ferrocene modified DNA, $Fe[(CN)6]^{3-/4-}$	10^5–10^{10} copies/mL	1.3×10^4 copies/mL	[54]
20	Breast cancer	CD63	CD63 aptamer	DPV	Doxorubicin	3.4×10^4 to 3.4×10^8 copies/mL	1.2×10^4 copies/mL	[55]
21	Prostate cancer	Prostate-specific membrane antigen	PSMA aptamer	DPV	$Ru(NH3)_6^{3+}$	1000–120,000 copies/µL	70 copies/µL	[56]

|							
22	Liver cancer	CD63	CD63 aptamer	SWV	Fe $[(CN)6]^{3-/4-}$	1×10^5– 5×10^7 copies/mL	1.72×10^4 copies/mL	[57]
23	Hepatocarcinoma, Breast cancer	miR-122	miR-122 complementary hairpin DNA	DPV	$Ru(NH3)_6^{3+}$	0.1 fM to 0.1 µM	53 aM	[58]
24	Cervical cancer, Breast cancer	miR-21	Complementary DNA probes	SWV	$[Fe(CN)6]^{3-/4-}$	10 aM to 100 fM	7.3 aM	[59]
25	Breast cancer	miR-21	miR-21 complementary DNA	SWV	Methylene blue	10 aM to 10^7 aM	3.04 aM	[60]
26	Coronary heart disease	miR-181	Oligonucleotide hairpin probe	EIS, CV, SWV	$[Fe(CN)6]^{3-/4-}$	10 fM-100 nM	7.94 fM	[61]
27	Breast cancer	miR-1246, miR-221, miR-375, and miR-21	Complementary DNA tetrahedron probes	EIS, CV, SWV	$[Fe(CN)6]^{3-/4-}$	10 fM t-100 pM	7.2 aM	[62]
28	Breast cancer	miR-21	miR-21 LNA probe	EIS, DPV	Methylene blue	10–70 fM	2.3 fM	[63]
29	Breast cancer	miR-21	miR-21 LNA capture probe	DPV	$[Fe(CN)6]^{3-/4-}$		67 aM	[64]
30	Prostatic cancer	miR-21	miR-21 DNA hairpin probe	EIS, SWV	$[Fe(CN)6]^{3-/4-}$	1 fM to 200 pM	0.4 fM	[65]
31	Breast, colon, oral cancer	miR-21	miR-21 biotinylated capture probe	DPV	$[Fe(CN)6]^{3-/4-}$	0.2–20 pM	1 pM	[35]
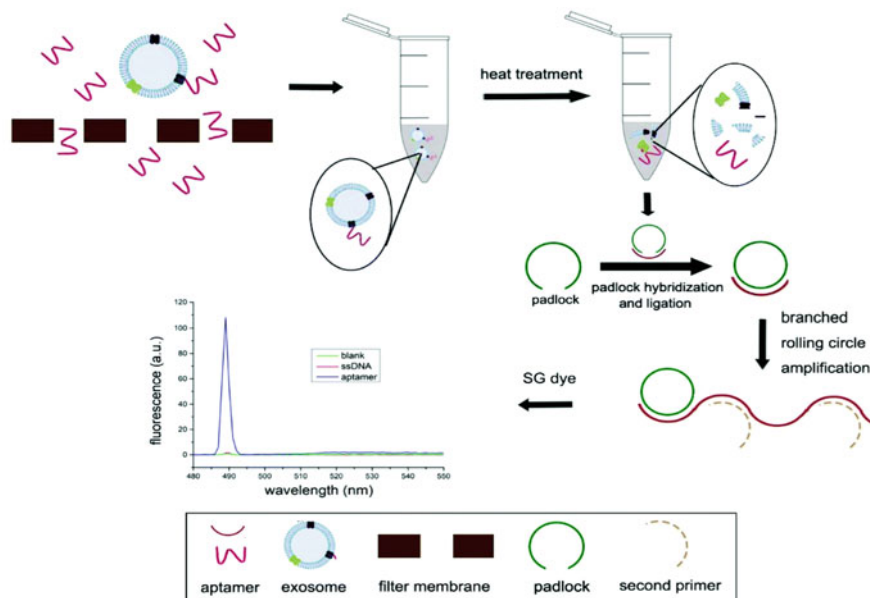
Fig. 7 Schematic illustration of branched rolling cycle amplification for gastric cancer exosome detection. Aptamer-bound exosomes are filtered and separated. Padlock DNA, ligase, polymerase, and primers are added for ligation and branched amplification steps. Reprinted from [70] with permission from The Royal Society of Chemistry

the linear DNA [69]. Annealing to the primer leads to the clipping of the two ends, which are then ligated with a ligase enzyme. This step is followed by the replication of the obtained circular DNA, yielding a continuous stretch of tandem repeats of a sequence complementary to the circular DNA [69]. In this process of signal amplification, there is an output signal increase of around 100–1000 fold, which is very important for the ultrasensitive detection of moieties in a complex biological system. Huang and the group developed a modified branched RCA method to amplify aptamers specific to gastric cancer cell-derived exosomal glycoprotein Mucin 1 (MUC1) [70]. The aptamers bound to isolated exosomes were separated with heat treatment and acted as a ligation primer against a linear 'padlock' DNA. For branched amplification, secondary primers were added and annealed to regions of the primary RCA product, followed by polymerization (Fig. 7) [70].

Proximity ligation assay (PLA) has improved traditional immunoassay technology that involves the simultaneous identification of different antigens (closely associated) on the same target [71]. The antigen targeting probes are conjugated with short specific oligonucleotide sequences. If the two probes reside in proximity, the complementary oligonucleotide strands hybridize and participate in rolling circle amplification, consequently taking part in target recognition [48]. Zhang et al. exploited similar technology to electrochemically detect human cervical cancer cell-derived EVs. They immobilized capture oligonucleotide fragments on the gold

working electrode. In order to capture protein tyrosine kinase (PTK) expressing EVs, PTK-specific S1 and S2 aptamer sequences were used. In the presence of target EVs, S1 and S2 form a duplex complex with a capture oligonucleotide sequence, which increased the electronegativity on the working surface and affected the increased detection signal [48]. This PLA-based electrochemical aptasensor specifically detected cancer-derived EVs with a detection limit of 6.607×10^5 [48]. Nevertheless, in such an amplification process, always there is a high chance of non-specific interaction between complementary RCA products.

Strand Displacement Reaction Amplification

Strand displacement reaction (SDR) amplification is another innovative strategy employing the kinetic characteristics of base complementarity for directing the displacement of a strand from a double helix, by a third strand with stronger binding kinetics [72]. One toehold-mediated SDR-based exosomal miRNA sensor was reported by Miao and Tang. The non-enzymatic strategy employed six DNA oligo-nucleotide sequences: probes A, B, C, D, E, and F [73]. The longer probe A partially hybridized with the shorter probe B and C to form a nicked duplex. Upon addition of miR-21 (target), miR-21 hybridized to the 5′-overhang (toehold) of probe A and replaced probe C in subsequent entropy-favored steps. The single-stranded region was then hybridized with probe D, which further displaced miR-21 and probe B [73]. The thus released miR-21 could further react with new probes A-B-C structures for many more cycles, so long as probe D was exhausted [73]. The cycles led to the release of a large number of probe B strands. Probe B then participated in down-stream SDR by opening a methylene blue-labeled hairpin probe E, on a gold electrode. In the final step, hairpin probe F hybridized partially to the opened probe E and displaced probe B [73]. The distance acquired between the electroactive methylene blue and the electrode because of the hybridization could be monitored by a reduction in voltammetric signal, due to the decreased oxidation rate of methylene blue [73]. Liu and the group developed a biosensor with localized toehold-mediated SDR strategy and DNA nanosheets (DNS) as labels for miR-21 detection [74]. Nine DNA strands: S1, S2, S3, S4, S5, S6, S7, S8, and S9 were designed to self-assemble into a nanosheet [74]. The annealing process was based on shared partial sequence complementarity between multiple strands, forming a DNA network. Double-stranded DNA redox intercalator methylene blue was loaded onto the DNS in high concentration [74]. The localized SDR setup also involved the formation of a polymeric T substrate (T_s) by chain hybridization, with L_1, L_2, P, and R strands forming a quadruple-stranded monomeric unit. L_1 had two terminal regions: I_1 and I_2, and L_2 had two terminal regions: I_1^* and I_2^* complementary to I_1 and I_2 respectively [74]). Lateral hybridization led to a bridge-like structure, while an R strand was bound to the mid-region of L_2, and a P strand to that of L_1. L_1 also had a mid-region sequence complementarity with the target miR-21, and the latter was capable of displacing and releasing the P strand in the reaction buffer [74]. Next, an F strand with a greater number of base pairings with L_1 and L_2 simultaneously was added which led to the release of both miR-21 and R strands. The released miR-21 could initiate further rounds of similar annealing and displacement reactions,
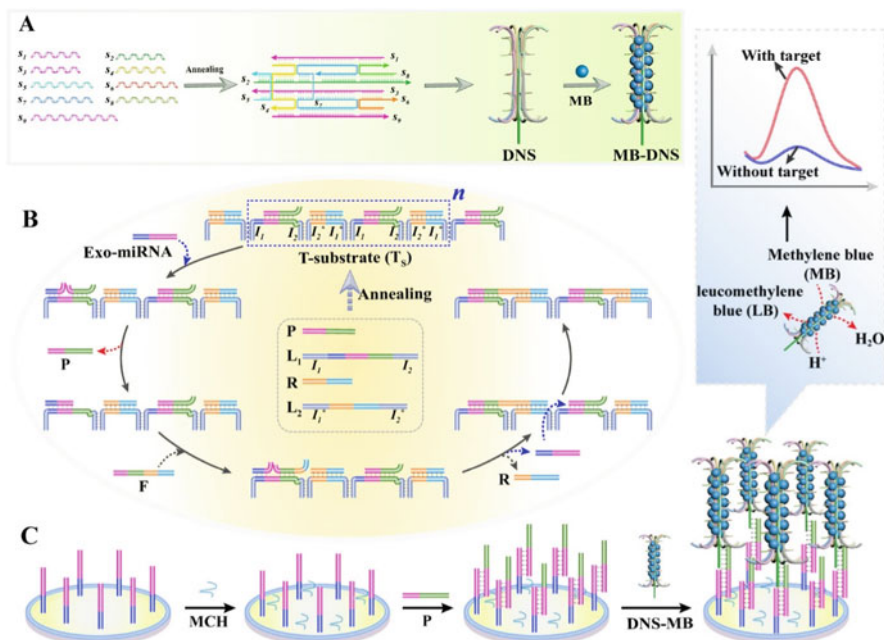
Fig. 8 Schematic illustration of the electrochemical biosensor for ultrasensitive detection of Exo-miRNA (miR-21); (**a**) assembly procedure of the methylene blue-loaded DNA nanosheet (DNS-MB); (**b**) the operation steps of the localized toehold-mediated strand displacement reaction; (**c**) binding of the methylene blue-loaded DNA nanosheets with the capture probes on the electrode via P strands. Reprinted from [74] with permission from the American Chemical Society

releasing a large number of P strands into the buffer [74]. The P strands were collected and hybridized into the immobilized capture probes on a gold electrode. In the final step, methylene blue-loaded DNS were hybridized to the single-stranded sticky end of the P strands, via the protruding end of the S_8 strand. Methylene blue was oxidized to leucomethylene blue to obtain the detection signal (Fig. 8) [74].

Shi and the group prepared an enzyme-based SDR strategy for miRNA detection. The target miRNA was 3′-end hybridized with a primer (primer 1) terminating in a nicking enzyme (Nt. AlwI) recognition site overhang [75]. Next, the Klenow fragment polymerase extended the 3′-ends of both primer 1 and miRNA. This was followed by Nt. AlwI nicking at the recognition site, and strand displacement by another intact primer 1, which could proceed for the next round of polymerization, nicking, and strand displacement steps [75]. The complementary miRNA strands released after nicking and strand displacement steps were hybridized with another primer (primer 2), with a similar nicking site overhang [75]. Subsequent polymerization, nicking, and strand displacement steps were undergone in a cyclic fashion. This dual cyclic process led to the exponential synthesis of miRNA target and complementary target sequences isothermally, from a very small starting amount of the miRNA [75].

Catalytic Hairpin Assembly

A modified form of SDR is the catalytic hairpin assembly (CHA), wherein kinetically stable hairpin structures are sequentially unwound via a triggered SDR with an added linear strand, to form an assembled structure [76]. Zhang and the group prepared a CHA-based biosensor specific for exosomal miR-181. Three hairpins (H1, H2, and H3) with partially complementary sequences were designed. Of these, H1 and H3 were biotin-labeled [61, 62]. SDR was sequentially triggered by miR-181, wherein it first unwound H1. The mid-section of H1 partially hybridized and opened H2, and the mid-section of H2 further hybridized with H3. H3 finally displaced miR-181, and the latter could initiate the next round of CHA. H1, H2, and H3 formed a T-shaped triple-stranded junction, which could associate with other similar hairpin junction structures via hybridization between 3'-H3 and 5'-H1 overhangs, to form long concatemers [61, 62]. In the detection step, the concatemers were captured via the 3'-H2 overhangs annealed to complementary probes on a gold electrode. Alkaline phosphatase (ALP) conjugated to streptavidin was captured with biotin labels and utilized for the conversion of α-naphthyl phosphate to the electroactive product α-naphthol [61, 62].

Compared with other isothermal amplification strategies, this technique comes out to be a suitable alternative to low concentration target detection. Although multiple step-based signal amplification increases the sensitivity significantly, it takes a huge time. In addition to that, stability is a big issue in such a sensor. To overcome those problems, ye zhang et al. designed DNA tetrahedrons assisted catalytic hairpin assembly (MDTs-CHA) based electrochemical biosensor for the specific detection of four breast cancer cell-derived EV-incorporated miRNAs (miR-1246, miR-221, miR-375, and miR-21) [62] (Fig. 9).

Howbeit, this kind of highly sensitive strategy requires multiple probes and a very complex signal amplification process, which limits their use in a practical scenario.

Hybridization Chain Reaction

Hybridization chain reaction (HCR) is another modified form of SDR, similar to CHA, comprising sequential partial hybridization of amplification probes to form ladder-like multistranded structures (concatemers). The detection signal is greatly amplified by labeling individual probes with a signaling tag [68]. For instance, Choi, Beck, and Pierce designed an HCR strategy for the detection of mRNA targets within whole-mount, intact zebrafish embryos with complementary DNA initiator probe sets and amplification hairpin pairs [77]. The hairpins coexisted stably until an initiator strand destabilized and opened the hairpins sequentially via toehold-mediated displacement [77]. A specific DNA initiator, say I1, contained a mid-region complementary to a region of the target mRNA and generated a 3'- and 5'- overhang/sticky end each upon hybridization. The 5'- sticky end was hybridized with H1 via its toehold, opening it [77]. The sticky end of H1 was further hybridized with H2, such that the sticky end of H2 was rendered identical to that of I1[77]. Thus, a chain reaction could be initiated indefinitely, till H1 and H2 were
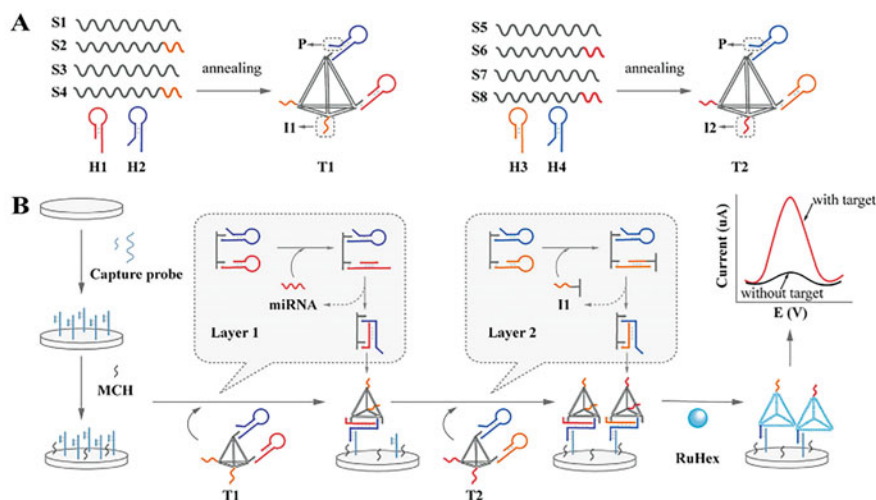
Fig. 9 Schematic illustration of electrochemical approach for the detection of breast cancer cell-derived exosome extracted micro RNAs. (**a**) Strand displacement assembly reaction was performed to synthesize T1 with S1-S4, H1, H2, and T2 with S5-S8, H3, H4 (**b**) Catalytic hairpin assembly based electrochemical biosensing steps on the surface of the gold working electrode. Reprinted from [62] Copyright (2021), with permission from Elsevier

exhausted. The authors of this study fluorescent-labeled the hairpins to amplify manifold the fluorescent signal (Fig. 10) [77].

In comparison with enzyme-based signal amplification, HCR show promises in terms of simplicity, stability, and efficiency in isothermal amplification. In recent times, HCR is highly popular in miRNA detection. Guo et al. fabricated miR-122 complementary hairpin DNA on the gold working electrode surface. In presence of HepG2 and MCF7 cell-derived EV extracted miR-122, the hairpin probe opened up to form a single straight hybrid [58]. Then helper DNA 1 and helper DNA 2 were used to initiate the HCR process (Fig. 11). Electroactive intercalation of the signal molecule $[Ru(NH_3)_6]^{3+}$ (RuHex) in the long-chain hybrid generated signal for sensitive detection [58].

Though HCR is a potential alternative to enzyme-based signal amplification it is restricted to dsDNA amplification only [60]. A recent novel isothermal nucleic acid amplification strategy has captured research attention that can amplify any arbitrary ssDNA in just one step with the help of a primer exchange reaction. Wang et al. designed a highly sensitive sensor using the concept of primer exchange DNA amplification reaction (PEDAR) and target-mediated cyclic strand displacement reaction (TMCSDR) [60]. As indicated in Fig. 12, the gold working electrode was modified with a primer probe that initiated the first round of signal amplification in presence of breast cancer cell-derived EV extracted miR-21 [60]. After a few cycles of TMCSDR, it generated many single-stranded primers. Then template probe was hybridized with the generated primer to start the second round of PEDAR signal amplification. Finally, the redox molecule methylene blue (MB) is bound
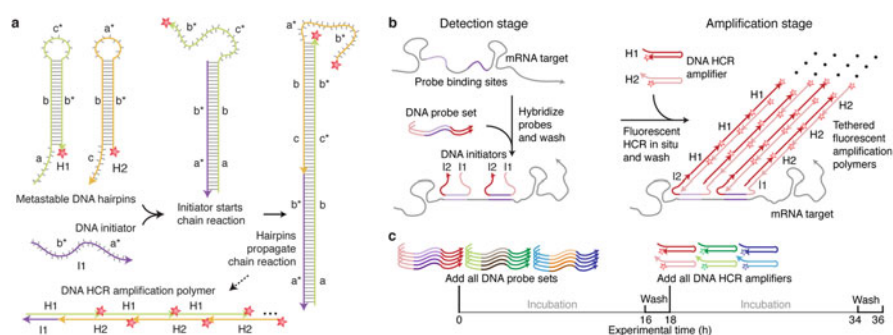
Fig. 10 Schematic illustration of in situ amplification via hybridization chain reaction (HCR). (**a**) HCR mechanism. Metastable fluorescent hairpins self-assemble into fluorescent amplification polymers upon detection of a cognate initiator. Initiator I1 nucleates with hairpin H1 via base-pairing to single-stranded toehold "a", mediating a branch migration that opens the hairpin to form complex I1-H1 containing single-stranded segment "c*-b*". This complex nucleates with hairpin H2 by means of base-pairing to toehold "c", mediating a branch migration that opens the hairpin to form complex I1-H1-H2 containing single-stranded segment "b*-a*". Thus, the initiator sequence is regenerated, providing the basis for a chain reaction of alternating H1 and H2 polymerization steps. Red stars denote fluorophores. (**b**) In situ hybridization protocol. Detection stage: probe sets are hybridized to mRNA targets, and unused probes are washed from the sample. Amplification stage: initiators trigger self-assembly of tethered fluorescent amplification polymers, and unused hairpins are washed from the sample. (**c**) Experimental timeline. The same two-stage protocol is used independently of the number of target mRNAs. For multiplexed experiments (three-color example depicted), probe sets for different target mRNAs (five probes depicted per set) carry orthogonal initiators that trigger orthogonal HCR amplification cascades labeled by spectrally distinct fluorophores. Reprinted from [77] with permission from the American Chemical Society

electrostatically with the amplified product to generate the electrochemical signal. In this label-free dual amplification process, they achieved a LOD of 3.04 aM that accurately discriminated even single-base mismatch. Such highly selective biosensors exhibited the significant potential to be used in point-of-care diagnosis [60] (Fig. 12).

DNA Nanomachines

Recently, several DNA nanomachines have been synthesized and designed to perform triggered motion, signaling, and conformation switching tasks. The nanomachines are categorized as DNA switches, walkers, motors, etc. [78]. A sensing platform reported by Zhao and the group utilized a DNA walker for the ratiometric release of signal probes from a small population of captured target EVs [54]. The designed DNA walker was composed of exosomal CD63-specific aptamers, immobilized on magnetic beads (MBs) via biotin-streptavidin linkage [54]. To discriminate MCF cell-secreted EVs from normal cellular EVs, dual recognition was conducted with exosomal EpCAM aptamers alongside CD63 aptamers. EpCAM aptamers were captured to the MBs only in the presence of target EVs, forming a sandwich complex with CD63- and EpCAM- aptamers. The EpCAM aptamer was extended to a swing arm and an Mg^{2+}-dependent DNAzyme,
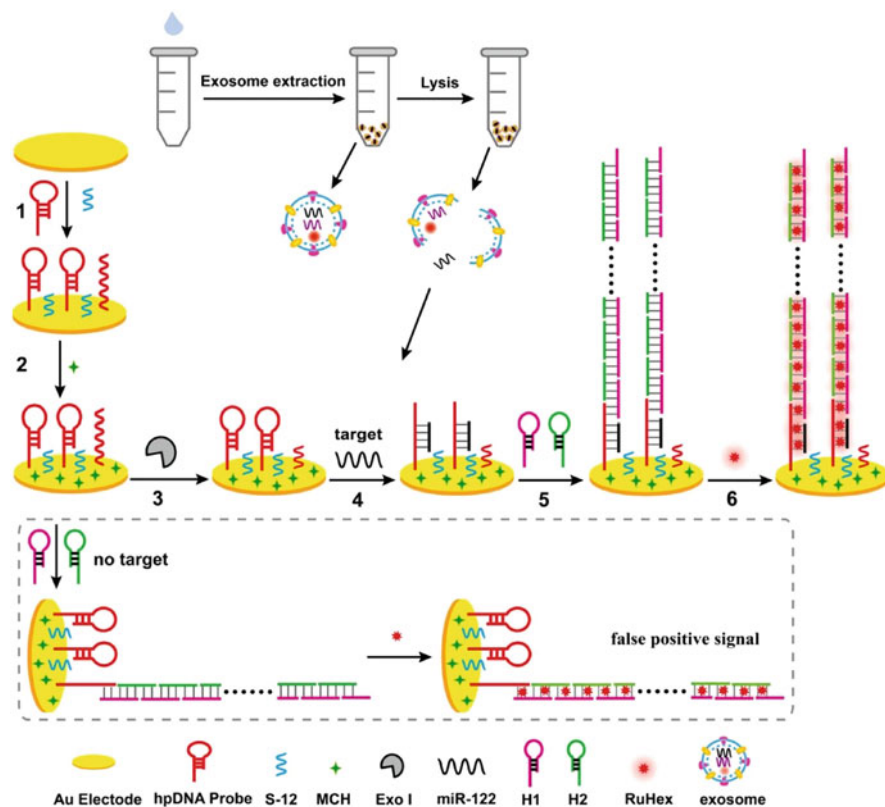
Fig. 11 Schematic representation of HCR-based electrochemical biosensor for the detection of breast cancer EV-derived miR-122. In the absence of target miR-122, H1 had the potential to trigger HCR reaction with single-stranded hpDNA which resulted in the false-positive signal. So as to reduce the false-positive signal, exonuclease 1 was used just before the incubation of miR-122. Exonuclease 1 hydrolyzed the single-stranded hpDNA in order to prevent any background signal from the sensor. Reprinted with permission from [58] Copyright (2021), American Chemical Society

flanked by A1 and A2 sequences [54]. The MBs were also functionalized with DNAzyme substrate sequences comprised of a ribonucleobase (rA) cleavage point and flanking P1 and P2 sequences. P2 and P1 hybridized to A1 and A2, respectively, when brought into proximity in the presence of EVs [54]. The DNAzyme then mediated a cleavage at rA and released P1 strands. This step destabilized and caused the melting of the hybridized structure. The cleavage and melting events propelled the free EpCAM aptamer-DNAzyme strands to hybridize into another DNAzyme substrate [54]. The released P1 strands were then used to unwind methylene blue-conjugated hairpin DNAs on a gold electrode. The exposed 3′-ends of the unwound hairpins were digested with a 3′-5′ exonuclease (Exo III), and P1 strands were further released for initiating multiple steps of hybridization and exonuclease
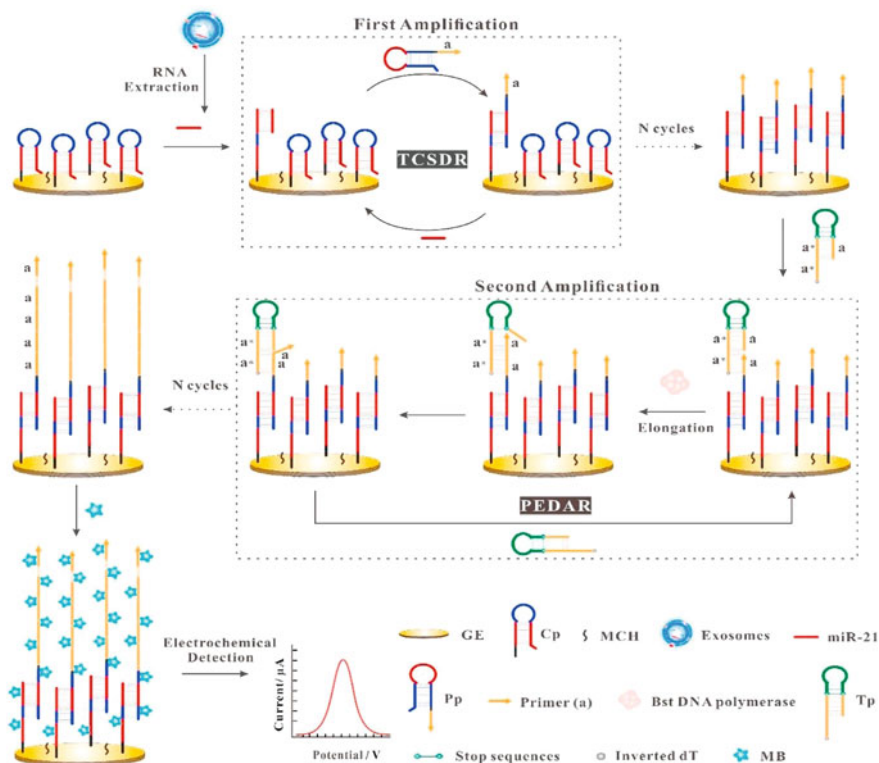
Fig. 12 Schematic representation of dual amplification electrochemical biosensor for the detection of breast cancer EV-derived miR-21. Reprinted from [60] Copyright (2021), with permission from Elsevier

activity. In the final signal generation step, several 5′-ferrocene (Fc) labeled DNA strands were hybridized into the partially digested hairpin DNAs on the gold electrode [54]. The ratiometric biosensing signal was obtained from the oxidation currents of both the electroactive labels: methylene blue and ferrocene (Fig. 13) [54].

6   Challenges and Future Directions

The inadequacies of conventional invasive diagnostic techniques coupled with recent advancements in molecular profiling of biofluids have led to the popularization of liquid biopsy as a tool for cancer diagnosis. EVs as novel analytes in liquid biopsies have triggered interest among researchers and exhibit potential as excellent biomarkers. Electrochemical sensing, associated with good selectivity and high sensitivity and the added features of low cost, rapid detection, minimum sample requirement, and simplicity, is an attractive alternative to conventional methods of
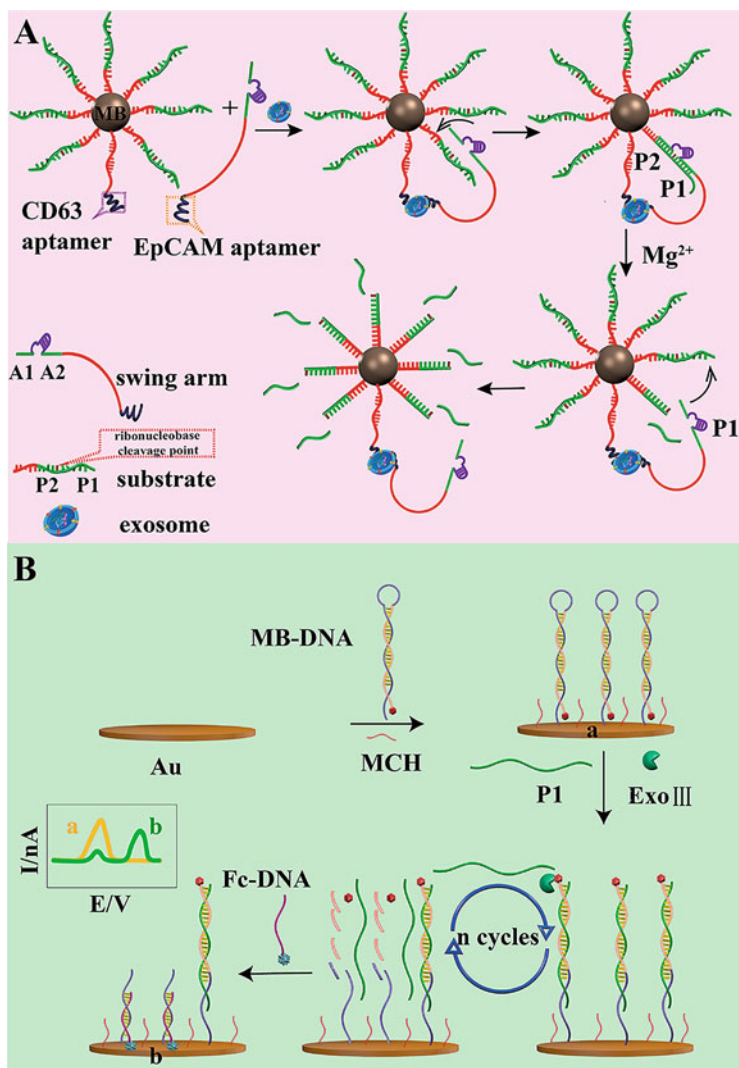
Fig. 13 Schematic illustration for the detection of exosomes through (**a**) 3D DNA walker amplification and (**b**) Exo III-assisted electrochemical ratiometric assay. Reprinted from [54] with permission from the American Chemical Society

EV detection and characterization. However, several bottlenecks could arise and need attention for facilitating the use of these techniques in the clinical setup.

Clinical samples are prone to biological variability; variations are often introduced during the collection and handling of biospecimens. Furthermore, detection of low abundant biomarkers is often challenging due to the inherent complexity of biological matrices and requires the use of appropriate sample extraction or

pre-treatment steps. The development of sensors based on a single marker while clinically appealing due to simplicity and low cost, may not capture the variability of disease through the population, which again lowers accuracy, sensitivity, and specificity. It is well accepted that even a small deviation in the sensor fabrication process including but not limited to electrode surface modification, sample pre-treatment, etc. may lead to inconsistencies in detection. Another challenge is the low signal-to-noise ratio and the presence of false positives which pose a serious threat to achieving high specificity. A number of excellent clinically useful sensors have been developed so far; however, reports on clinical trials conducted globally for validation of these fabricated sensing devices are lacking, thereby raising the question of reproducibility.

EV research has emerged as an attractive noninvasive diagnostic option for cancer in the last decade. Not only in diagnosis but these natural stable vesicles show promise in drug delivery, regenerative, and personalized medicine. Rose Johnstone first used the word "exosome" in 1983 [79]. Initially, these small vesicles were reviewed as "garbage bags," just to remove the waste products from cells. It took almost four decades to understand the pathological and structural aspects of EVs in cellular communication. In 2013, Dr. Rothman, Dr. **Schekman, and Dr. Südhof were honored with the Nobel prize for reporting the vesicular transporting machinery in the human system** [80]. Since then there is an unexpected growth in EV research. According to journal citation reports published in 2016, a total of 51, 913 times the keyword "exosome research" was used in correlation with any analytical disorders [81]. The "PLOS ONE," "JOURNAL OF BIOLOGICAL CHEMISTRY," and "SCIENTIFIC REPORTS" were the highest holding journals for EV research according to the report published in 2016 [81]. Initiation of a few dedicated societies for EV research such as the *Journal of Extracellular Vesicle* (JEV), *International Society of Extracellular Vesicles* (ISEV), and *European Journal of extracellular vesicle* (ESEV) appeared to motivate newborn ideas in this field. There are a large number of databases for instance "Vesiclepedia" (www.microvesicles.org), ExoCarta (www.exocarta.org), EV-TRACK (www.evtrack.org), exRNA Atlas (www.exrna-atlas.org), etc. were started to encourage to store and access the EV related information. According to the UTSPO database in 2016 in total 524 US patents were granted involving EV [81]. Various clinical and pre-clinical trials are ongoing in this particular field and most of them are associated with a cancer diagnosis. Many governments and nongovernment agencies have been involved in granting funding for EV research. Understanding the impact of EV research numerous companies have started to commit to this area. Companies include Aethlon Medical, Inc., Exopharm Ltd., Lineage Cell Therapeutics, Exosome Diagnostics Inc., Clara Biotech, Sphere Fluidics, ThermoFisher Scientific Inc., etc. The global EV market is expected to reach $2.28 billion at a CAGR of 18.8% by 2030 [82]. Although many new perspectives have emerged related to EVs for clinical translation, still standardization of qualitative and quantitative EV research is still a challenging job. According to MISEV, the clinical application of EV depends on various pre-clinical factors such as coagulation agent, storage time,

and isolation steps [5]. Several biochemical and biophysical variables have been found to alter the pathophysiological profile of EVs.

Recent progress in the sensitive sensing of EVs by electrochemical biosensors has promised to bridge the challenges. The real-time accurate point-of-care (POC) diagnosis of the target analyte is very important especially in highly populated countries like India to reduce the massive burden on the healthcare sector [83]. Various electrochemical biosensors have achieved a very low detection limit by manipulating sensing parameters. With the recent integration of microfluidics and nanotechnology, it is envisioned that the issues associated with sample pre-treatment and signal amplification can be addressed significantly. The extensive use of nanomaterials has improved the sensitivity of the platforms to a considerable extent. Multiplexed detection is another prominent feature that can influence the development of electrochemical sensors.

7   Conclusion

EVs have amassed an ample amount of research recognition in the recent era due to their capacity to carry disease-specific biomarkers from one cell to another. An elevated number of tumor-specific EVs emerged as an excellent circulating liquid biopsy biomarker for cancer. To explore these aspects, standardized EV analysis techniques are essential. Many interesting biochemical, and biophysical techniques are unable to quantify tumor-derived EVs with high sensitivity. Scientists are constantly trying to advance the science, technology, and innovation (STI) sector for the development of accurate POC medical devices. With the immense pressure that has stretched in the healthcare sector after the COVID-19 pandemic, it is relevant to develop low-cost, rapid, sensitive POC medical devices for high prevalence diseases. The performance of an effective biosensor not only relies on the stability of the biorecognition molecule aptamer or antibody but also on a sensitive signal detection method to quantify cancer-derived EVs at very low concentrations. Herein, we presented the advantage of electrochemical biosensors and their potential implications in EV sensing. Further, the newborn ideas and signal detection strategies with electrochemical biosensors were discussed in this review. The majority of electrochemical proof-of-concept studies for cancer-derived EV quantification are listed in Table 2. Despite the considerable advances in these fields, most of the sensors require traditional strategies like ultracentrifugation and chromatography for the isolation of EVs from human bioliquids. Moreover, it is well reported that the biochemical properties of EVs get affected by different isolation strategies. For that reason, it is very important to standardize the EV isolation method, before detection. On top of that, the vague knowledge of EV storage conditions, and stability faces difficulty to translate individual research insights into the clinical market. We believe that more research and recommendations will enable us to design an electrochemical 'extracellular vesicle on chip' POC medical device in near future.

Conflicts of Interest The authors declare no conflict of interest.

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