Past five-year statistics report reveals that, 7.8 million women were breast cancer (BC) diagnosed, at the end of 2020, marking it as the world’s most ubiquitous cancer. A higher occurrence is noticed in women beyond 40 years of age who had higher lost disability-adjusted life years (DALYs) compared to other cancer types. The BC begins as premalignant lesions, develops to the pre-invasive stage of ductal carcinoma in situ (DCIS) and ends as invasive ductal carcinoma (IDC). Nearly, 20%–25% of newly diagnosed BC in the past 5 years represents DCIS and up to 40% has the chance to progress as IDC. All DCIS are considered as stage 0 BC, and are found in the breast milk ducts as a proliferation of mammary gland neoplastic cells that have not invaded into the surrounding stroma.

DCIS Stages, Grades and Incidence

The stages of DCIS are (i) Ductal hyperplasia or “overgrowth”—where more number of cells compared to normal growth is present in the breast tissues, (ii) Atypical ductal hyperplasia—presence of hyperplasia starting to take abnormal appearance, (iii) DCIS—too many cells having the features of cancer yet present only inside the duct, (iv) DCIS microinvasion—fewer cancer cells starting to penetrate through the wall of the duct (also called as stage I BC) and (v) IDC - the cancer cells have spread out of the breast duct and is considered as stage I-IV (see Scheme 1). Additionally, the DCIS is graded as lower (grade I), moderate (grade II) and high (grade III) as shown in the Scheme 1. Low grade DCIS is not dangerous at the initial stage, but if left untreated there are high chances of developing high and/or intermediate grade and progressing to IDC. Therefore, diagnosis and treatment of BC at DCIS stage is the utmost need in the present situation which leads to long-term survival rate (10-year survival rate exceeds 95%) and abridge economical and psychological distress.

Conventional BC screening for DCIS and reason for alternative diagnosis method.—Till date, mammography of the breast tissues via biopsy is the gold standard DCIS diagnosing tool. However, limitations such as poor sensitivity (~67%) for dense breast tissues, invasive means of sample collection, effect of radiation, about 10%–30% of undetected cases and high false-positive and false-negative reports due to technical/human error signifies the necessity of alternative reliable techniques for DCIS diagnosis. Magnetic resonance imaging (MRI), the alternative to mammography is also radioactive, expensive, intensive, requires skilled technical assistance. Furthermore, microcalcifications (MC) identification for BC screening on DCIS that is usually done via mammogram, MRI and ultrasound imaging may not be suitable for real-time clinical application and needs series of biopsies to confirm whether, the DCIS is benign or malignant, which again is a nerve-racking protocol. This reveals the unmet necessity to develop non-invasive, rapid, sensitive, reliable and non-radioactive method to diagnose DCIS. In this context, a “liquid biopsy,” which is the detection/quantification of DCIS biomarkers which are present in the body fluids of the patients’ will be the best suited alternative to overcome the above-mentioned limitations. These biomarkers can be used to obtain the information about DCIS occurrence and its nature of invasion at most early stage. Numerous sensor platforms, exclusively the electrochemical biosensors utilizing enzyme-modified, DNAs immobilized, aptamers based, nanomaterials and composites modified, etc. have become an evolving alternative method due to their easy operating nature, on-site detection possibility, affordable instrumentation, smallness, and its robustness of developing point-of-care screening and diagnostic devices for the detection of BC biomarkers. Nonetheless, the electrochemical detection of MCs which is composed of calcium oxalate and hydroxyapatite cannot be a direct reliable diagnosis. However, to achieve sensitivity, specificity, improved test efficiency, shortened analytical time, decreased sampling volume, reduced cost and reliable diagnostic results it is highly preferable to quantify and determine the potential biomarkers responsible for the DCIS condition via liquid biopsy. Nevertheless, most of the reports have been focussed on the BC biomarkers which have been recognized as metastatic BC biomarkers, which are found at stages I-IV.

Diagnosis via Blood Based Biomarkers.—In this context, the blood-based biomarkers are considered crucial for the screening of BC in its tumorigenesis stage before it has spread beyond the primary site. However, the well-known cancer antigen (CA15–3, CA27–29), and cancer embryonic antigen (CEA), blood tumor markers for BC, are still non-endorsed by the European Group on
Tumor Markers (EGTM) and the American Society of Clinical Oncology (ASCO) guidelines for BC screening even though approved by FDA due sensitivity issues and the lacking confirmation for their significance in early-stage BC control. Other significant DCIS biomarkers include microribonucleic acids (miRNAs), lipids, and the proteins p16, COX-2, Ki67, in addition to HER-2, estrogen and progesterone. Among the above-mentioned DCIS biomarkers, the miRNAs are considered as minimally invasive liquid biomarkers identified in blood (plasma/serum) that acts as a diagnostic tool/desirable biomarker to detect cancer at very early stage accurately. The blood biopsy is a simple procedure where it can be done plasma exosome extraction can be avoided within 2 h of total analysis duration using very low sample volumes needed, which indicates its high possibility to be extended to clinical application. On this basis, numerous research has been reported for the single miRNA detection using varied electrochemical biosensors where electrochemistry technique reduces the false positive results due to interference and ambiguous signals to a greater extent, reveals its highly useful nature for physiological and pathological diagnosis over the conventionally used Real-time PCR (qRT-PCR) qRT-PCR. The qRT-PCR even though is widely used powerful tool for miRNA assay and DCIS screening tool that could achieve high sensitivity involves tedious procedures and expensive primer sequences that restricts its further critical applications (see Scheme 2).

For quite some time the RNA molecules were not agreed to be used as blood-based biomarkers, due to the high nucleic contents found in plasma, however miRNAs were identified to be stable in samples of particular tissues. Moreover, one miRNA quantification is not adequate enough to diagnose not only DCIS condition, but for any disease nature and type. Therefore, the assaying of multiple miRNA markers from one sample is necessary for accurate detection and higher specificity. Only handful of research has been reported thus far on the multiplexed and simultaneous detection of only two or three miRNAs using biosensors for early BC detection from the body fluids. To the best of our knowledge, there is no systematic review conducted on the electrochemical biosensors for multiplexed DCIS miRNAs detection. This systematic review aims to throw limelight on multiplexed miRNAs responsible for the BC screening at early stage reported using non-invasive, rapid diagnostic and screening tool in bringing down the socio-economic sufferings involved in BC and its treatment. Besides, particular emphasis is given on risk of bias (ROB) involved in the reported studies on multiplexed miRNA electrochemical biosensing. Through the methodology quality assessment, the reported studies are discussed for its eligibility to be extended as future diagnostic tool for DCIS. Finally, the limitations and new developments for the current expectations in the field of developing alternative tool for the mammography and qT-PCR have been briefed at the end of this review. We are certain that this review will promote more pioneering research on electrochemical biosensors for DCIS diagnosis.

Materials and Methods

Search scheme and keywords.—A comprehensive search was conducted using the chosen keywords in December 2021 and February 2022 in PUBMED, Science Direct, Scopus, MEDLINE, Cochrane Library, National Centre for Biotechnology Information, and Google scholar. The keywords used for the search are: (“electrochemical biosensors”) AND (“stage 0 breast cancer” OR “ductal carcinoma in situ” OR “DCIS”) AND (“multiple miRNAs” OR “multiplexed miRNA detection” OR “multiplexed miRNA breast cancer biomarker” OR “miRNA BC biomarkers” OR “simultaneous miRNA detection” OR “breast cancer screening” OR “simultaneous BC biomarkers detection” OR “miRNA based BC screening” OR “breast cancer miRNAs detection”) AND (“early detection” OR “early BC detection” OR “BC screening miRNA biomarkers” OR “early stage breast cancer miRNA screening” OR “BC screening biomarkers” OR “BC screening biosensors”) An additional search was conducted in February 2022 in Citation searching, conference proceedings and abstracts and thesis abstract in line with the protocol.

Inclusion and exclusion criteria.—Only the studies focussed on simultaneous and multiplexed detection of miRNA breast cancer markers using electrochemical biosensor and/or with comparison of miRNA biomarker detection between healthy subjects and cancer subjects were selected for this systematic review, and all the other types of studies were excluded. Original research articles, published in English language from 2012–2022 were included for review. Researches published before 2012, book chapters, encyclopaedia, mini reviews, systematic reviews, review articles, and in other languages were excluded in this systematic review.

Study selection.—A 4-member reviewer’s team were involved in the article selection process. The collected research titles and abstracts from the above-mentioned electronic database were screened independently by 2 reviewers (S.S. and H.A.). The other 2 reviewers (G.B and C.V) screened the articles published from the conference proceedings, citation searching and thesis abstracts. The mentioned above inclusion and exclusion criteria were used for articles selection. The articles title and abstract with chosen keywords were scrutinised before going through the full text of the article. If any difference of opinion arose among reviewers, discussion and consensus meeting was conducted to arrive at final conclusion.

Scheme 1. (A) Stages and (B) Grades of Ductal Carcinoma In-situ (DCIS).
Data extraction.—The Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) guidelines 2020 were followed to extract the data from the selected articles for reporting this systematic review.

Risk of Bias (ROB) assessment criteria.—The assessment of ROB of the published original articles using standard guidelines is not available in electrochemistry/science professions. Therefore, the quality assessment criteria was set based on the electrochemical sensing parameters and validation analysis to evaluate published articles for the eligibility to be extended as POCDs after discussion in the reviewer’s team. However, we assessed the quality of studies included by 14-item quality scoring adopted from scientific literature and further modified to fit for electrochemistry studies given in Table I. The ROB involved in the reports has been classified as, the articles fulfilled, (a) < 5 out of 14 criteria are described as high ROB articles, (b) 5–9 out of 12 criteria is represented as medium ROB articles and (c) those reported ≥9 out of 14 criteria is identified as low ROB articles.

Results and Discussion

Expression of DCIS miRNAs in body fluids.—The miRNA biomarkers that are up and down regulated in the body fluids which could be DCIS diagnostic biomarkers (differentiating between cancer and DCIS condition) in agreement with the published research articles and reports involving pathology and biology, of DCIS are listed in Table II.

Identification of studies from the databases.—The study design was established based on the PRISMA guidelines 2020 (see Fig. 1). Initially, the identification of the articles was done using the databases, registers, websites and citation screening. In the comprehensive electronic search done from seven databases, 3,94,899 articles were excluded before screening due to the ineligibility to participate in the scope of this review article. Out of 4,07,717 articles screened from databases and registers and 5,89,402 and 500 via websites and citation searching respectively, 4,06,183 and 5,89,467 were excluded. This exclusion also includes duplicates from different sources. A second eligibility screening was done based

| S. No. | Criteria                                                                 | Point |
|-------|--------------------------------------------------------------------------|-------|
| 1     | Electrode preparation complete description with optimization              | 1     |
| 2     | Samples prepared by the operator as per standard procedure               | 1     |
| 3     | Report of sensitivity with relative standard deviation                   | 1     |
| 4     | Report of limit of detection with relative standard deviation            | 1     |
| 5     | Reproducibility sensing tested under two different electrochemical instruments | 1     |
| 6     | Repeatability sensing tested by different operator (at least two)        | 1     |
| 7     | Stability report sensing tested by different operator (at least two)     | 1     |
| 8     | Interference testing for the biomarkers related to the specific disease condition (specificity) | 1     |
| 9     | Detection from the human healthy real samples                            | 1     |
| 10    | Detection from patient real samples                                      | 1     |
| 11    | Mechanism of interaction between the electrode surface and the analyte   | 1     |
| 12    | Validation with standard biochemical method                               | 1     |
| 13    | Blinding of the operator during characterization/graph plotting           | 1     |
| 14    | Description of sample size calculation in real sample/human sample testing | 1     |

Table I. The ROB assessment criteria and the bias point allocation used in this study for the literature reports’ eligibility to be extended as diagnostic tool.
The following reasons: 1: single biomarker detection, 2: biosensors that do not involve electrochemical detection, 3: treatment of DCIS, 4: imaging and other techniques, 5: review articles discussing single miRNA biomarker, 6: research articles discussing pathology, histochemistry, biology and clinical aspects of miRNA biomarkers of DCIS, 7: non-BC miRNAs detection in spite of multiplexed sensing were excluded. Out of 1973 total assessed number of articles, only 17 articles were included for this review.

Table II. miRNAs responsible for the DCIS condition expressed in the body fluids.

| Condition | miRNA expressions |
|-----------|-------------------|
| DCIS      | Upregulated       | Downregulated |
|           | Plasma            | Serum         | Plasma          | Serum          |
| miR-21    |                   |               | miR-155         |               |
| miR-141   |                   |               | miR-19a         |               |
| miR-10b   |                   |               | miR-181b        |               |
| miR-155   |                   |               | miR-24          |               |
| miR-150   |                   |               | miR-526 b       |               |
| miR-140 (exosome) | | miR-519 a     |               |
| miR-145+miR-451 |       | miR-571       |               |
| miR-376c  |                   |               | miR-139–3p      |               |
| miR-801   |                   |               | miR-206         |               |
| miR-424   |                   |               | miR-193a-3p     |               |
| miR-184   |                   |               | miR-140         |               |
| miR-409–3p|                   |               | miR-125b        |               |
| miR-376a  |                   |               | let-7 family    | miR-132       |
| miR-148 b |                   |               |                 |               |
| miR-190   |                   |               |                 |               |
| miR-127–3p|                   |               |                 |               |
| miR-200a-c|                   |               |                 |               |
| miR-429   |                   |               |                 |               |
| miR-182   |                   |               |                 |               |
| miR-183   |                   |               |                 |               |

Figure 1. Articles screening and study design according to the PRISMA guidelines 2020.
Intriguing mechanism of the electrochemical biosensors for multiplexed biomarker detection.—In order to develop a multiplexed electrochemical biosensor for miRNAs detection, the most important step is the electrode modification based on varied complementary oligonucleotide probes (DNAs) for each miRNA targets and/or co-immobilization of oligonucleotides is the crucial step and the intrinsic linear structure of oligonucleotide makes its preparation step difficult. Label-free electrochemical technique which is highly selective, sensitive, stable and easy to prepare nature can be considered as a potent tool for the multiplexed cancer biomarkers detection. The electronic conductivity change at the modified electrode surfaces with and without the target miRNAs-probes hybridized with the redox capture tags is the electrochemical signal noticed (see Fig. 2). This systematic review covers electrochemical biosensors and its related analysis such as chemiluminescence, photoluminescence and fluorescence combined with conventional electrochemical detection methods such as: cyclic voltammetry (CV), differential pulse voltammetry (DPV), square wave voltammetry (SWV), amperometry and linear sweep voltammetry (LSV) reported during 2012–2022 exclusively for the simultaneous and multiplexed miRNAs detection which are expected to be present in the DCIS stage.

Comparative electrochemical performances for miRNA-141 and miRNA-21 electrochemical detection.—Two of the miRNAs namely miRNA-21 and miRNA-141 which are reported to be upregulated in the human plasma due to DCIS has been evaluated by few authors. The highest performance in terms of limit of detection (LoD) has been reported by Mohammadniaei et al. using an unique amplification strategy introduced by combining MXene (Ti3C2Tx) and a duplex-specific nuclease (DSN) resulting in attomolar and quantification for duplex miRNAs on a single platform and demonstrated its detection from total plasma. The MXene with higher surface area is loaded with gold nanoparticles (AuNPs) and drop casted on a dual screen-printed gold electrode and co-immobilized with DNA target probes. Biofouling resistance and excellent electrochemical signals were obtained on this modified electrode due to homogeneously deposited AuNPs within MXene sheets and the thiol-Au bonding features. The LoD values of 204 aM and 138 aM respectively were obtained for miRs 21 and 141 for concentrations ranging from 500 aM to 50 nM (Table III). To prove the concept to be extended as practical biosensor, the authors coupled the modified nanocomposite with a 96-well device which sensed the two miRs and demonstrated for three plasma samples from the cancer patients.

For a femtomolar detection of miRNAs simultaneously, a modified electrode was developed by Tian et al. displayed a novel a paper based sensing device using metal organic framework (MOF) conjugated bio-probe consisting of two layers. The silver nanowire acted as conducting support for the paper based sensor on which the MoS2/AuNPs was modified which extended high surface area to hold on the capture oligonucleotides thereby opening pathway to show the electrochemical current signal. In order to further increase the target capture ability, the PtCuMOFs were utilized. Thus, the MoS2/AuNPs/AgNW paper electrode surface with the PtCuMOFs held to the oligonucleotides and the redox tags methylene blue (MB) and ferrocene (Fc) generated electrochemical signals. A 10 μl aliquot of miRNA sample were dropped on to the modified paper electrode at 37°C for 2 h and further addition of hemin to form G-quadruplex structure (G4) for hydrogen peroxide (H2O2) catalysis were accounted for the electrochemical signal. This sensor showed better sensitivity, LoD and linear range compared to other fluorescence, chemiluminescence, CV and SWV of other miRNA targets (miR-21, 122, 223, 27b, 210 and 196 a) with 0.1 fM LoD when the electrochemical luminescence (ECL) reported elsewhere by other authors showed 1.51 fM and the SERS showed 2.75 fM indicating the superior performance of the MOF used in this work. Alternatively, Wang et al., used nitrogen-doped hollow carbon nanospheres with large pores (pNHCSs) synthesised using green microwave assisted methodology. The three-dimensional structured nanospheres incorporated with nitrogen increased the enzyme loading and power output of 325 ± 0.6 μW cm⁻². Based on a dual-fuel-driven substrate, the authors displayed a self-powered biosensor for duplex miRNAs detection using electrochemical

Figure 2. Illustration of multiplexed miRNA electrochemical sensing mechanism.
| S. No | Probe Target | Method | Sensitivity | Linear range | LOD | Ref No |
|-------|--------------|--------|-------------|-------------|-----|--------|
| 1     | Generic neutravidin modified electrode | miRNA-21 and miRNA-141 | SSWV | 4.5 and 3.7 nA/pM | 0.5–1000 pM and 50–1000 pM | 0.3 pM and 10 pM | Azzouzi et al., (2019) [45] |
| 2     | Thiol-modified, redox species-labelled hairpin probes on the gold sensing electrode | miRNA-141 and miRNA-21 | SWV | — | 5.0 fM to 50 pM | 4.2 fM and 3.0 fM | Yang et al., (2014) [47] |
| 3     | AuNPs/GQDs/GO coated SPCE | miRNA-21, miRNA-155, and miRNA-210 | SWV | 3.5 μA/pM | 0.001–1000 pM | 0.04 fM and 0.33 fM | Pothipor et al., (2021) [48] |
| 4     | TBApy-MeCoFeCOOH | miRNA-155 and miRNA-122 | DPV | — | 0.01–1000 pM | 6.7 fM and 1.5 fM | Cao et al., (2019) [49] |
| 5     | R-R1/TDN/depAu/GCE | miRNA-21 and miRNA-155 | DPV | — | 0.1 fM — 10 nM | 18.9 aM 39.6 aM | Xu et al., (2020) [50] |
| 6     | Silica Nanolabels | miRNA-21 and miRNA-141 | CV | — | 0.02 — 120 pM | 6.3 fM and 8.6 fM | Peng et al, (2016) [51] |
| 7     | DOPE/DOTAP/AuNP | miRNA-124a | DPV | 1.7 μA g⁻¹ M⁻¹ | 500 aM—1 pM | 0.1 fM | Ghazizadeh et al., (2018) [52] |
| 8     | RP1 & RP2@PbS, CdS QDS / CP1 & CP2@MB | mir-155 and mir-10b | SWV | — | 50 fM—30 pM 50 fM—1050 pM | 12 fM 31 fM | Zhu et al., (2014) [53] |
| 9     | DNA1/Fe₃O₄ NPs/Thi DNA2/Fe₃O₄ NPs/Fc | miR-141 and mirRNA-21 | DPV | — | 1 fM to 1 nM | 0.44 fM and 0.46 fM | Yuan et al., (2017) [54] |
| 10    | CdS:Mn NCs film/ GCE | miRNA-21 and miRNA-155 | ECL | — | 5.0 fM to 500 pM | 1.51 fM and 1.67 fM | Peng et al., (2017) [55] |
| 11    | AuNPs and CdS Nanogears | miRNA-21 and miRNA-155 | ECL | — | 50 pM to 0.5 fM | 0.16 fM and 0.33 fM | Zhang et al., (2017) [56] |
| 12    | MoS₂/AuNPs-PtCuMOFs | miR-21, miR-141 | SWV | — | 1 fM to 1 nM | 0.1 fM | Tian et al. (2019) [57] |
| 13    | SPCE/rGO/P2ABA/AuNPs | miRNA-16 | DPV | — | 1 fM—10 nM 0.25 fM 3.58 fM and 0.98 fM | Pimalai et al., (2021) [38] |
| 14    | GOD/ADH co-functionalized pNHCS/AuNp/CP electrode | miR-21 and miR-141 | EIS | — | 10⁻⁶—10⁻¹⁰ M | 0.1 fM and 4.0 fM | Wang et al., (2018) [58] |
| 15    | UIO-66-NH₂ | let-7a and miRNA-21 | DPV | — | 100 to 1000 nM | 3.6 fM and 8.2 fM | Chang et al., (2019) [59] |
| 16    | AuNP-decorated MXene-Ti₃C₂Tx/ SPGE | miR-21 and miR-141 | DPV | — | 500 aM to 50 nM | 204 aM and 138 aM | Mohammadniaei et al., (2020) [60] |
| 17    | CA 15-3/BSA/anti-CA 15-3/DAP-AuNPs/P3ABA/2D-MoS₂/GO/SPCE | miRNA-21/MCH/capture DNA-21 probe/TB-AuNPs/P3ABA/2D-MoS₂/GO/SPCE | DPV | 4.75 μA Uml⁻¹ | 0–500 Uml⁻¹ 0–14 U ml⁻¹ | 0.14 U ml⁻¹ | Pothipor et al., (2022) [37] |

SWV—Square wave voltammetry, SSWV—stripping square-wave voltammetry, GOD—Glucose oxidase, ADH—alcohol dehydrogenase, NHS—N-hydroxysuccinimide, LSV—Linear Sweep Voltammetry, DPV—Differential pulse voltammetry, Fe-CHO—ferrocene carboxaldehyde, Thi—thionine GQD—Graphene Quantum Dots, GO—Graphene oxide, AuNPs—Gold Nanoparticles, SPCE—Screen printed Carbon Electrode, TBApy-MeCoFeCOOH—tetra(4-carboxyphenyl) pyrene and melamine, TDN—tetrahedron DNA nanostructure, GCE—Glassy Carbon Electrode, RP1 & RP2—Receptor Probe 1 and 2, PbS & CdS QDs—Lead Sulphide and Cadmium sulphide Quantum dots, CP1 & CP2—Capture probe 1 and 2, MB—Magnetic beads, CdS:Mn NCs—Cadmium sulphide and manganese Nanocrystals, SPCE/rGO/P2ABA/AuNPs—reduced graphene oxide/poly(2-aminobenzylamine)/gold nanoparticles.
impedance spectroscopy (EIS). The achieved detection limits were 0.1 fM and 4.0 fM for the miRNAs 21 and 141. An economical, green and handy biomedical sensors has been fabricated by the authors.38 However, the reported bioanodes and cathodes inspite of exhibiting high power, the ability to detect the biomarkers was less compared self-powered biomedical sensors has been fabricated by the authors.38 However, the reported bioanodes and cathodes inspite of exhibiting high power, the ability to detect the biomarkers was less compared to the MXene based electrodes.

Slightly higher LoD values compared to the above studies for the miRNAs 21 and 141 were revealed by Yang et al. through redox labels and DSN-assisted signal amplifications. The sensor contains self-assembled thiol and redox mediator-labelled hairpin probes on the Au electrode. The hybridization between the target RNA and surface modified DNA duplexes resulted in duplex signals with LoD of 4.2 fM and 3.0 fM. Impressively, the sensor also showed excellent specificity between human prostate carcinoma (22Rv1) and breast cancer (MCF-7) cell lysates miRNAs.47 Even though the thiol and nano gold particles are involved in this work, the biological interaction between the electrode and the analyte seems to be lesser than the thiol functionalized Fe3O4 nanoparticles and AuNPS loaded MXene biosensors reported by Yuan et al. and Mohammadniaei et al. discussed earlier.

In addition, Feng et al. reported detection of the above-mentioned target miRNAs using the CV combined ECL biosensor array developed on a self-made screen-printed carbon electrode through co-immobilized multiple capture probes using doped silica nanolabels.54 The poly-L-lysine (PLL) and ruthenium immobilized silica were used as electrochemiluminescent signal tags along with the AuNPS modified on the SPCE. The Ferrocene (Fc)-labeled hairpin DNA (Fc-HDNA1 and Fc-HDNA2) immobilized on Ru-SiO2@PLL-Au was used as CV signal tag and ECL quenching material. The targets were then spiked on to the electrode surface where hybridization takes place and separates Fc away from Ru-SiO2@PLL-Au which resulted in decreased CV current of Fc. The ECL “signal-on” and “signal-off” mechanism was possible due to the dual-signal ratiometric indicators and exhibited LoD values of 6.3 and 8.6 fM for miRNA-21 and miRNA-141, respectively. For practical application efficacy demonstration, the authors simulated the healthy human blood serum real sample. However, the detailed real sample preparation and standard addition procedures were not provided and there is a lack of detection from the patient samples. Moreover, the LoD were comparatively lesser to that of the other electrochemical biosensors discussed earlier.

However, all the above-mentioned electrodes needs multiple probes for detecting multiple miRNAs, but the only research provided and there is a lack of detection from the patient samples. However, the detailed practical application efficiency demonstration, the authors simulated the healthy human blood serum real sample. However, the detailed real sample preparation and standard addition procedures were not provided and there is a lack of detection from the patient samples. Moreover, the LoD were comparatively lesser to that of the other electrochemical biosensors discussed earlier.

Comparative electrochemical performances studies reporting electrochemical detection for miRNA-21 and miRNA-155.—The highest performance in terms of LoD was achieved for miR-21 and 155 in the work by Xu et al. where a novel circle capture DNA probe anchored DNA nanostructure with tetrahedron shape was used as a detection probe (see Fig. 4). The potentials of detection where with +0.46 V and −0.31 V obtained in CV. The MB and Fc acted as redox capture probes along with the DNA nanostructure which attributed to interference elimination and enhanced detection sensitivity. This biosensor revealed LoDs of 18.9 aM and 39.6 aM for miRNA-21 and miRNA-155 in addition to appreciable specificity and good detection efficiency from the MC-7 cancer cells.50 The three-dimensional tetrahedron DNA nanostructure exhibited well-regulated structure alignment and less nonspecific adsorption and enhanced target catch. Overall, the redox probes Fc and MB involves in increased current signals for the targets which is detected as highly electro-conductive peak current responses using DPV technique.

Pothipor et al. identified non-labelled electrochemical biosensor with AuNPs graphene quantum dots and graphene oxide modified three-array SPCE which takes the second positionin terms of LoD values of 0.04 fM and 0.33 fM for miRNAs-21 and 155 respectively under SWV in addition to another miRNA-210. Three different redox targets namely, anthraquinone (AQ), methylene blue (MB), and polydopamine (PDA) were used to capture three different miRNAs (21, 155 and 210) due to the large specific surface area and high electronic conductivities of the gold, quantum dot and graphene oxides. The developed biosensor reveals simultaneous sensing with sensitivity values of 3.5, 2.9 and 1.8 μA/fM for the three miRNAs and also demonstrated selective recovery from human serum samples.48 The significance of the design of the reported biosensor is that it is simple and without any labelling molecules, less time for the fabrication and detection, low cost, and reduced assay procedure.

Zhang et al. designed a dual miRNAs-triggered DNA nanogears56 as enzymeless electrochemical luminescent biosensor to detect miRNAs (21 and 155). The CdS QDs and AuNPs were used as key components and allowed to roll between the DNA nanogears A and B. The ECL energy transfer between AuNPs and CdS QDs were noticed and when the target miRNAs come into the line, the ECL quenching takes place due to Förster energy transfer. The ECL signals for the two miRNAs showed LoD values of 0.16 fM and 0.33 fM. This enzymeless technique is a new trend to use ECL detection with one lumiphore for multiple miRNAs quantification. The report illustrated reproducibility only for few numbers of electrodes and moreover, it was efficient in detecting the miRNA from the cancer cells. However, it would be highly efficient to detect the miRNAs upregulated in the blood samples directly from the cell lines which may be the suitable method to develop POCDs. An important idea to be noted in this work is the usage of DNA label free modified electrode for the miRNAs sensing which again is advantageous in practical applications in terms of storage and stability aspects.

An interesting dual miRNA powered DNA walking machine which can walk to and fro was developed by Peng et al.35 via enzyme-free biosensor worked on the principle of ECL energy transfer which is dependent on the distance between the capture and the target molecules. They demonstrated DNA walker to move forth and ECL quenching when the miRNA-21 come closer between the AuNPs and Mn3+ doped CdS nanocrystals. Whereas, when miRNA-155 is dropped in, the walker moved back automatically, where (for 500 pM) for miR-141. The percentage errors for interassay lied from ~5 to 12%. To note: For real industrial application a commercial biosensors which possesses, an inter-assay error below 15% which is considered to be good. Therefore, compared to all the above discussed literatures, the single pot assay method seems to be highly useful for multiplexed miRNAs detection for developing POCDs for diagnosing BC at early stage.
surface plasmon resonance was noticed due to the increased distance between AuNPs and CdS:Mn NCs, and thus an increased ECL signal was observed. The LOD achieved were 1.51 fM and 1.67 fM, respectively. However, the recoveries of the miRNAs were discussed only with the phosphate buffer solution and no practical demonstration with the real body fluids was reported in this work.

Pimalai et al. reported highly sensitive electrochemical biosensors to detect three miRs-155, 21, and 16 on a reduced graphene oxide (rGO), poly(2-aminobenzylamine) (P2ABA) and AuNPs. The hollow metal NPs act as the tag labels on the modified electrodes. An anti-deoxyribonucleic acid (DNA)-RNA hybrid [S9.6] antibody was used for target detection where the hybridization between varied DNAs and RNAs in two steps. Firstly, the signal enhancement was achieved due to the nanocomposite before the antibody immobilization. Next, the modified electrode was connected with the capture DNAs with different labelled metal ions. The developed electrochemical biosensors was highly stable, sensitive and selective with linear range of detection from 1 fM to 10 nM and LoD of 0.98 fM and 3.58 fM, respectively. The proposed electrochemical biosensor proved its capability for multiplexed BC biomarkers detection in normal human serum sample to illustrate its potential candidacy for BC screening.38

Comparative electrochemical biosensing performance for other miRNAs (210,122,221,124a,16,279,375, let 7a) and CA15-3.—Cao et al. proposed electrochemical aptasensor utilizing covalent organic framework using shell-encoded AuNPs as signal labels with silver nanoclusters and copper oxide respectively named as AgNCs@AuNPs and Cu2O@AuNPs to detect the miRNAs 155 and 122. The 1,3,6,8-tetra(4-carboxylphenyl) pyrene and melamine (TBAPy-MAE COFe COOH) polymerization formed due to condensation process was used to synthesize the covalent organic framework which was then anchored with single-strands of the respective miRNAs through hybridization formed with the complementary capture aptamers. The developed aptasensor showed LoDs of 6.7 and 1.5 fM, respectively, and linear range of 0.01–1000 pM, and appreciable recovery of miRNAs from the simulated human serum samples.49 However, in concern with the diagnosis of particular diseased condition, multiple cancer biomarkers detection cannot be considered. Therefore, the detection of specific condition biomarkers is highly needed for prototypes for specific applications.

Another multiplex detection system developed with two separated working electrodes by Ghazizadeh et al. involved DOTAP-DOPC liposomes, chimeric probes, p19 caliper molecule, and the RNA competitor structural hybrid for the detection of miRNAs...
The DOTAP-DOPE liposomes, which were spherical in nature and had cationic charge hybridized with the (T-M-linear, Stem) and the miRNAs stabilized the sensor. When p19 was added, for miR21, the electrode showed a signal and for the other two, the signal was off, which revealed excellent specificity and LoD of 0.1 fM. The detection time taken for the proposed electrode is 1 h. The electrode finds its efficacy in detecting one of the DCIS miRNA biomarker (miR-21). Usage of p19 protein in this study is notable as p19 turns the unstable system to a stable system when connected with firm RNA-miRs.

Zhu et al. developed an electrochemical method simultaneous miRNAs in a single-tube experiment which contains no labels and no PCR. The ligase chain reaction (LCR) which showed high base-mismatch selectivity combined with the electrochemical QDs barcodes are the main theme of the system reported. The PbS and CdS QDs acted as reporting probes, and two capture probes co-immobilized on the magnetic beads (MBs) formed the conjugate which acted as the electrode surface that is incubated at 90°C. The miRNAs samples incubated with the conjugates, was then added with T4 DNA ligase. The SWV signals for the two miR-155 and 27b were achieved through two different QDs barcodes from the MB conjugates. LoD values of of 12 fM and 31 fM for 50 fM–30 pM and 50 fM–1050 pM concentrations of 155 and 27b were achieved. The full quantification process took less than 70 min, and showed satisfactory recovery values of miRNAs quantification from human sera. Amidst the scarcity in the electrochemical multiplexed identification of miRNAs, this work may be considered as an alternative to the PCR technique.

Pimalai et al. reported highly sensitive electrochemical biosensors to detect three miRs-155, 21, and 16 on a reduced graphene oxide (rGO), poly(2-aminobenzylamine) (P2ABA) and AuNPs modified electrodes which resulted in 0.25 fM LoD for miRNA-16 and demonstrated its quantification in human serum. A label-free AuNPs/GQDs/GO/SPCE with polydopamine (PDA) redox species revealed sensing with 1.8 sensitivity and also demonstrated selective recovery from human serum samples. The simple design of the reported work with no labelling molecules, reduces fabrication cost, and operational complexity of the assay.

For the first time, Pothipor et al. reported an electrochemical biosensor for simultaneous detection of two different kinds of BC biomarkers, namely cancer CA 15–3 and miRNA-21. Healthy human serum shows less than 30 U ml⁻¹ of CA 15–3 and increased concentration is a symptom of high risk BC, therefore its detection from human serum is important in BC screening and/or diagnosis (see Fig. 5). The nanocomposite electrode consists of polyaminobenzylamine, grapheme oxide and two-dimensional molybdenum selenide modified on SPCE designed with two working areas which was further functionalized individually with 2,3-diaminophenazine and toluidine blue incorporated with AuNPs as redox probes. The electrode was then co-immobilized with anti-antibody for CA 15–3 and copy DNA for the miRNA-21. Superior electronic conductivity and high surface-to-volume ratio of the nanocomposite, are the key reason for more antibody and capture probe loading which further influenced enhanced peak current and selectivity for the target components. The LODs of 0.14 U ml⁻¹ and 1.2 fM for CA 15–3 and miRNA-21, respectively were achieved in this work and further was demonstrated for the target detection from simulated serum samples which is very important as mentioned in the introduction section of this review that along with miRNAs, other proteins and hormones which needed to be tested for the DCIS condition makes the sensor suitable to be an alternative diagnostic tool to mammography.
Chang et al. identified a new electrochemical biosensor which could detect let-7a and miRNA-21 using functionalized metal-organic frameworks prepared by using porous UIO-66-NH₂, and double stranded DNA. The hybridization with target miRNAs triggers the generation of RNA-DNA complexes that separated from MOFs and released the electroactive dyes which are loaded with the porous UIO-66-NH₂. The detection limits down to 3.6 fM and 8.2 fM, respectively were achieved for the targets and demonstrated to detect the miRs simultaneously from the simulated healthy human serum samples detected using DPV. In continuation with the discussion on the electrochemical performance of various electrodes for eligibility to be extended as an alternative diagnostic tool to PCR and mammography, the ROB scoring has been analysed for all the above discussed studies. Table III reveals the comparative significance of the reported articles in terms of possibility of being POCD for early BC screening in practical.

**Summary of the ROB assessment.**—Risk of bias, is the assessment made to identify to which extent a study design and its procedures is free from all the possible bias which affects the outcome of the study. Thus, assessing the risk of bias of a study is crucial to identify the true effect of the test conducted for a particular application. In this systematic review, the ROB assessment is done to provide a meaningful and valid conclusion by assessment of the methodology quality of the included studies in the review to bring out a new diagnostic tool alternative to gold standard mammography for early BC screening especially for the DCIS condition. Therefore, in this review the criteria set for ROB assessment (Table I) focuses mainly on the key experimental parameters and studies to be carried out for electrochemical biosensing for multiplexed miRNAs detection which can be further extended to clinical trials.

As mentioned in the Table IV, out of 17 studies, 16 studies lie in the medium ROB limit, which indicates that a conclusion cannot be arrived based on the available data (data insufficient for development of POCDs based on the reported studies). The complete description of sensor preparation and its optimization (Criteria 1 and 2) are necessary for reproducibility in terms of quantification value to achieve transparency and to verify the performance of reported work and have potential for mass production. Criteria 3–8 and 11 and 12 (sensitivity, LOD, reproducibility, repeatability, stability, specificity, mechanism of interaction and validation with gold standard instrument) are the important tool/device parameters to demonstrate the proposed sensor is robust and to extend it to the market. Criteria 9 and 10 are included in ROB assessment as they are highly significant to make us to understand how the electrode acts specifically and without any fouling in the presence of real samples and exhibit the quantification details. Blinding of the operators (criteria 13) are considered to minimize the risk in the manipulation of physico-chemical and electrochemical characterizations. The determination of sample size calculation (criteria 14) is necessary to make a meaningful conclusion and to generalize the findings of the study to the whole study population. Overall, the ROB is to be taken seriously for all the individual research for the concept of...
Table IV. Comparison table for the ROB identification of the literature reports available till date on multiplexed early BC miRNAs detection via electrochemical biosensors.

| Article                          | Criteria | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | Score | ROB |
|----------------------------------|----------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|-------|------|
| Azzouzi et al., (2019) [45]      |          | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 10 | Low   |
| Yang et al., (2014) [47]         |          | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 5   | Medium|
| Pothipor et al., (2021) [48]     |          | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 7   | Medium|
| Cao et al., (2019) [49]          |          | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 6   | Medium|
| Xu et al., (2020) [50]           |          | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 5   | Medium|
| Feng et al., (2015) [51]         |          | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 5   | Medium|
| Ghazizadeh et al., (2018) [52]   |          | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 5   | Medium|
| Zhu et al., (2014) [53]          |          | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 6   | Medium|
| Yuan et al., (2017) [54]         |          | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 6   | Medium|
| Peng et al., (2017) [55]         |          | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 5   | Medium|
| Zhang et al., (2017) [56]        |          | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 5   | Medium|
| Tian et al. (2019) [57]          |          | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 6   | Medium|
| Pimalai et al., (2021) [38]      |          | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 6   | Medium|
| Wang et al., (2018) [58]         |          | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 6   | Medium|
| Chang et al., (2019) [59]        |          | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 7   | Medium|
| Mohammadniaei et al., (2020) [60]|          | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 7   | Medium|
| Pothipor et al., (2022) [37]     |          | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 7   | Medium|

Multiplexed miRNAs detection using electrochemical biosensors to be considered as future diagnostic tool as an alternative to mammogram for early BC diagnosis.

Limitations, and future perspectives.—Till date, only few research has been published for the miRNAs electrochemical detection in aim to detect BC at the early stage (DCIS). The authors claim that two or three of the miRNAs responsible for early-stage BC has been successfully detected using proposed electrodes. Still, there is scarcity in developing a diagnostic tool for DCIS diagnosis and/or screening based only on two or three biomarkers. Moreover, out of 17 studies 16 studies lie in the medium ROB level and need further extensive investigation on multiplexed sensing of more number of miRNAs. Unfortunately, the reported studies lack validation with standard biochemical analysis which is the crucial step for the biosensor to be extended for practical POCDs for DCIS.

Current knowledge in developing POCDs.—Off late, the wearable POCDs have demonstrated an intense role in analytical chemistry due to its rapidity and capability for the real-time monitoring of environment and human health related analytes such as toxic chemicals, electrolytes and biomarkers. In line with this, lab-made potentiotstat and the stochastic sensors involving machine learning (ML), artificial intelligence (AI) for data analysis will be highly suitable for the multiplexed miRNAs detection which is still lagging behind in the early diagnosis of BCs.

Another important criterion for any sensors to enter into the market is the inter- and intra-assay repeatability, reproducibility and stability analysis which is highly lacking in the electrochemistry along with miniaturisation, multifunctional, wearable/implantable, stable, flexible, highly sensitive and specific, easy to fabricate, integrated with small devices and affordable. Electrochemical biosensors though considered as low cost and time consuming, which takes very less sample volumes, handy, user-friendly with appreciable specificity and detection limits possess serious limitations such as not reporting the detection in a greater number of real samples (healthy control and patients) fails to compete the available POCDs for glucose, cholesterol etc. Therefore, more extensive research is needed to detect maximum number of miRNAs responsible for the early BC conditions especially DCIS with excellent validation amidst minimally invasive sample collection to be proposed as a diagnostic tool for early BC detection. To achieve such targets future of biosensing should go in hands with the development of microarray electrodes integrated with AI. Thus, multiple miRNAs assaying could be used as potent biomarkers for the screening of BC at DCIS stage and are expected to act as an auxiliary diagnosis method for mammography and PCR.

Conclusions

Taken together, the findings of this systematic review, it is identified that electrochemical biosensors for early BC diagnosis is still at the infant stage and needs more works on the development of POCDs utilising the miRNAs as potential biomarkers which should involve inter- and intra-assay analysis and validation with standard biochemical tests and with a large sample of BC patients. The ROB assessment signifies that with most of the medium quality studies reported till date, it is not sufficient to bring out to a conclusion that duplex or triplex miRNA detection cannot be an alternative diagnostic tool to that of mammogram and q-PCR for early BC screening for DCIS stage. Therefore, there is an urgent need for high quality data analysis for multiplexed miRNAs electrochemical biosensing.
