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Review Article

Comprehensive insights into pathogenicity and emerging CRISPR-integrated biosensors for the early detection of breast cancer biomarkers

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ABSTRACT

Breast cancer is a significant healthcare burden and leading cause of female mortality globally. This necessitates for efficient, accurate, and early diagnosis of breast cancer. Presently employed diagnostic strategies suffer from major limitations in terms of false positives, false negatives, invasive procedure, and low sensitivity. Hence, there is a urgent need of development of diagnostic procedures which can address these limitations. Biosensors, with their high sensitivity represents a leap forward in development of non-invasive and rapid detection tool. Further integration of nanotechnology and clustered regularly interspaced short palindromic repeats (CRISPR) system have multi-fold increased the detection capabilities of these biosensors. This review article discusses breast cancer's pathogenicity, highlighting disease progression, risk factors, biomarkers, and genes associated with tumorigenesis. This review also explores and critically examines the traditional detection strategies. Furthermore, the advancement in biosensing techniques have also been discussed along with the role of nanomaterials in enhancing the competence of biosensors. The review critically examines the advantages, challenges, and applications of CRISPR-based biosensors for the detection of breast cancer. This comprehensive review article underlines the need for continuous innovation in breast cancer diagnostics to enhance patient outcomes.

1. Introduction

Breast cancer is the most common malignancy associated with women, with the leading cause of female cancer deaths worldwide. According to the global cancer observatory (GLOBOCAN) estimates of 2022, it is ranked second in terms of incidence, with nearly 2.3 million new cases, and fourth leading cause of global cancer mortality with 6.9 % of all cancer deaths [1]. The increasing prevalence is attributed to various lifestyle as well as reproductive risk factors such as, early menarche, late menopause, fewer children, declining breastfeeding, hormonal therapies, oral-contraceptives, consumption of tobacco and alcohol, obesity, and lack of physical activity [2]. Genetic factors, including mutations in *BRCA1* and *BRCA2* genes have significantly increased the risk of breast cancer. At the molecular level, mechanisms like mutations in tumor suppressor genes and oncogenes, epigenetic alterations, and disruption of hormonal regulatory pathway can also lead to abnormal cellular growth and proliferation leading to breast cancer [3].

Despite advancements in the technology for screening and treatment, the complexity of breast cancer makes early detection a challenge. Several traditional methods employed for breast cancer detection often lack in sensitivity and specificity for dense breast tissues, could show false positive and false negative results, expensive, requiring specialized equipment and expertise, and are generally invasive [4]. Consequently, there is an urgent need to develop diagnostic techniques that are highly accurate and precise, can offer early detection along with minimal invasiveness.

Biosensing techniques have emerged as a powerful tool for cancer detection, offering several advantages over conventional detection methods. These biosensing methods are cost-effective, technically simple, non-invasive (liquid biopsies and breath analysis), rapid, and have miniaturization as well as multiplexing capabilities [5]. Amongst them, biosensors incorporated with clustered regularly interspaced short palindromic repeats (CRISPR) technology, presents a promising frontier in early and accurate breast cancer diagnosis. Integration of CRISPR technology with the biosensors have addressed many of the limitations

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associated with conventional biosensors, such as detection range and interference issues, making them a promising advancement in breast cancer diagnostics [6].

In this review article, we discussed pathogenicity associated with breast cancer, disease progression, classification, risk factors, biomarkers, genes involved, traditional detection methods and new age biosensing diagnostic techniques with special emphasis to the emerging role of CRISPR-based biosensors. Further, we have also discussed their advantages, applications, challenges, and explored the future prospects in improving breast cancer outcomes and subsequently reducing healthcare burden.

2. Breast cancer

2.1. Breast cancer statistics

Breast cancer is the foremost health concern and cause of morbidity and mortality among women [7]. Fig. 1 depicts incidence and mortality data of year 2022 for breast cancer in the major continents as provided by International Agency for Research on Cancer (IARC) [1].

2.2. Risk factors associated with breast cancer

Breast cancer is a multifactorial disease with the exact cause not being defined with certainty. A complex interplay of genetic, environmental, and lifestyle factors contributes to its pathogenicity [8]. Understanding and timely detection of these risk factors would enhance the patient outcomes. These risk factors could be categorized as the one which can be controlled and the ones which can't be controlled as shown in the Table 1 [4,9].

Table 1
Major risk factors associated with breast cancer.

Risk factors which can be controlled	
Breast feeding	Lack of breast feeding
Consumption of oral contraceptive pills	Affects female hormones
Lifestyle factors	Consumption of alcohol and tobacco, lack of physical exercise, and night work
Nutrition and food choices	• Processed and packaged food consumption • Excessive consumption of fats and low consumption of proteins • High consumption of animal meat • Increased chances of obesity after menopause • Less consumption of fresh vegetables and fruits • Less intake of phytoestrogens (isoflavones, lignans)
Risk factors which can't be controlled	
Hormonal and reproductive factors	• Menarche at early age • Menopause at late age. • First reported pregnancy at a late age (after 30 years of age) • No pregnancies
Physiological factors	• Increased risk from 35 years of age • Family history of breast cancer • Past occurrence of breast, ovarian and endometrial cancer • Appearance of benign changes in the breasts, • Exposure to ionizing radiation
Previous disease conditions	• Oncogenic virus (e.g., Epstein–Barr) • Diabetes • High blood pressure • Changes in thyroid gland

2.3. Breast cancer progression

Breasts are prone to various pathological conditions at all ages starting from puberty to menopause. Inflammations, traumatic injury, benign and malignant tumors are some of the most common pathologies associated with breast [10]. Breast cancer originates in the breast tissue, mainly in the ductal cell linings (ductal carcinoma) or in the lobules (lobular carcinoma) [11]. It primarily affects women, although in some rare cases men can also develop the disease [12]. The progression of breast cancer through different stages are shown in Figs. 2a and 2b.

2.4. Classification of breast cancer

Breast cancer is often considered as a highly heterogeneous disease and is classified in four important subtypes including, Luminal A,

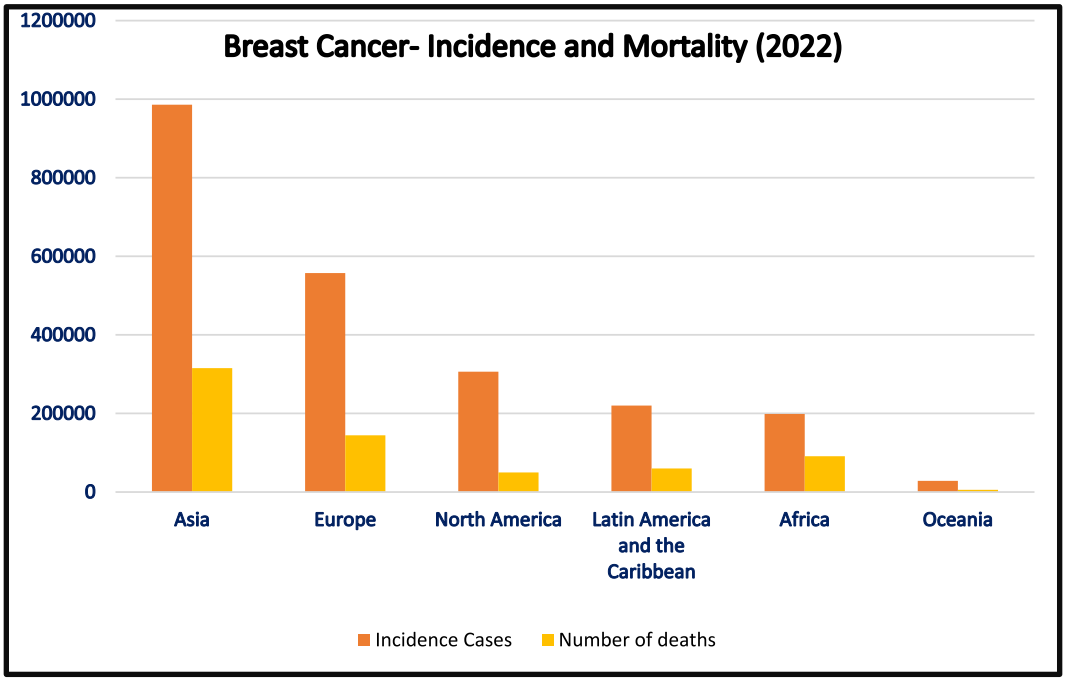

Fig. 1. Graphical depiction of breast cancer incidence and mortality data of year 2022 for breast cancer in the major continents (IARC).

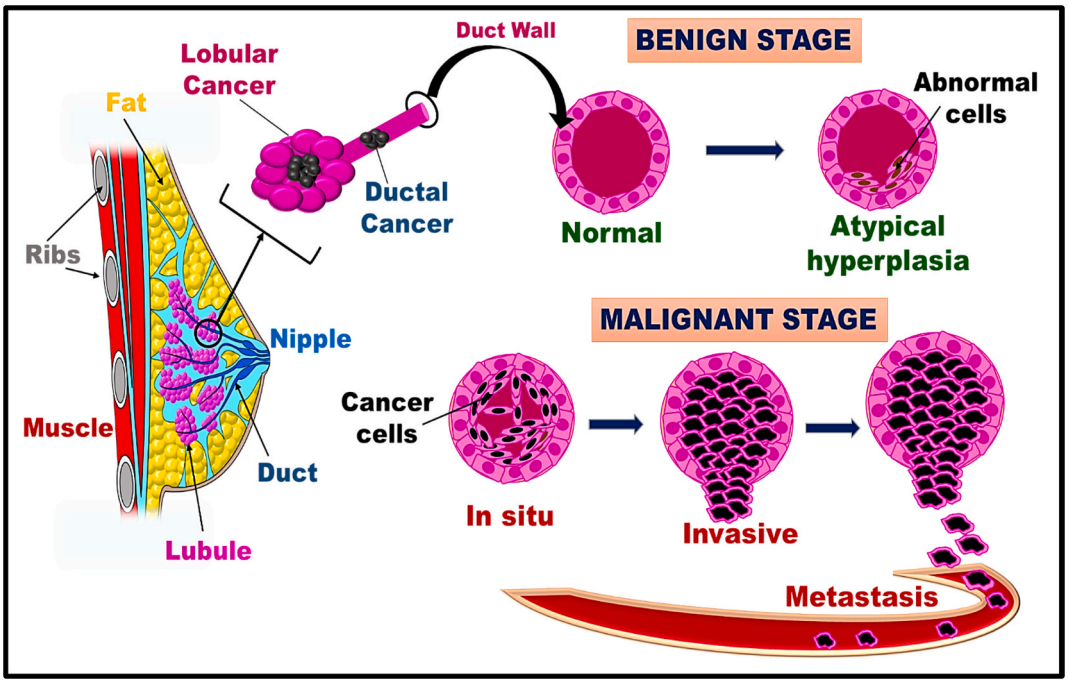

Fig. 2a. Diagrammatic representation of progression of breast cancer through benign and malignant stages.

Stage	Stage 1	Stage 2	Stage 3	Stage 4
				
Tumor Size	< 2 cm	2-5 cm	> 5 cm	Any size
Lymph node	Not affected	Few armpits affected	> 10 nodes may be affected	Any number of nodes may be affected
Characteristic	Confined to breast area	Confined to breast area	Confined to breast area	Spread to any other part of the body
Survival Rate	100 %	87 %	61 %	20 %

Fig. 2b. Diagrammatic depiction of stages of breast cancer depending on the tumor characteristics.

Luminal B, HER2 (human epidermal growth factor receptor 2) positive, and triple-negative breast cancer (TNBC) [13]. This classification is mainly on the basis of the presence or absence of certain receptors that influence tumor behaviour and subsequent treatment as depicted in supplementary Table 1.

Luminal A subtype is most common and accounts for majority of the breast cancer cases. It has slower growth rate as compared to other subtypes and have better prognosis with higher survival rates [14]. Some of the key RNA/DNA biomarkers associated with luminal A subtype includes estrogen receptor (ER), progesterone receptor (PR), antigen kiel 67 (Ki-67), miR-19a, and miR-205 [18]. Ki-67 is a proliferation marker that is low in luminal A tumors hence, correlating with good prognosis of luminal A subtype as compared to others. Luminal B is more aggressive than luminal A breast cancer with higher growth rate and a greater chance of recurrence [19]. The important RNA biomarkers associated with it are miR-15b-3p, miR-149-5p, miR-182-5p, miR-193b-3p, miR-200b-3p, and miR-342-3p, 5p [20]. Some of the other genetic biomarkers for luminal B subtype includes solute carrier family 10 member 6 (*SLC10A6*), signal recognition particle 9 (*SRP9*), DSN1 component of MIS12 kinetochore complex (*DSN1)*, and Rac GTPase-activating protein 1 (*RACGAP1)* [21]. HER-2 positive breast cancer has overexpressed levels of HER2 protein which results in increased cell growth and division hence, making cancer more aggressive [16]. Some of the important reported biomarkers for HER-2 positive breast cancer includes miR-125b, miR-146a-5p, mi-R-181d, miR-195-5p, miR-101-5p, mir-518a-5p, miR-19b-2-5p, miR-1237-3p, miR-29a-3p, miR-29c-3p, miR-106a-5p, and miR-744-3p [20]. TNBC subtype is considered most aggressive due to its rapid growth, high grade as well as increased chance of spreading and recurrence. It lacks targeted receptors, making hormone therapy and other therapeutic strategies difficult [22]. The important RNA/DNA biomarkers for TNBC subtype are connective tissue growth factor (*CTGF*) and phosphate and tensin homolog (*PTEN*), miR-455-3p, miR-107, miR-146b-5p, miR-17-5p, miR-324-5p, miR-20a-5p and miR-142-3p [20,23].

2.5. Biomarkers for breast cancer

Biomarker is a measurable biological indicator associated with certain disease, condition, state or process. These can be molecules, genes, proteins, or cells found in bodily fluids or tissues that can be used to assess health, diagnose diseases and its progression [24]. Biomarkers can be, diagnostic biomarkers for identifying disease, prognostic biomarkers for prediction of disease outcome, and predictive biomarkers for predicting response to a specific treatment [25].

In the context of disease such as breast cancer they could be majorly classified as blood-based and non-blood-based biomarkers [26]. This classification is further explained through Fig. 2c. With the advances in scientific research, there are novel biomarkers being discovered for improved personalized and precision medicine approaches hence, improving diagnosis and patient outcomes.

2.6. Breast cancer pathogenicity

Breast cancer involves a complex interplay of genetic mutations in various signalling pathways that control the growth, proliferation, survival of the cells. Alterations in various genes like overexpression of oncogenes and down regulation of tumor suppressor genes are often associated with breast cancer progression. Various genes involved along with their functions, associated pathways, and their role in the development of breast cancer are explained in the Table 2.

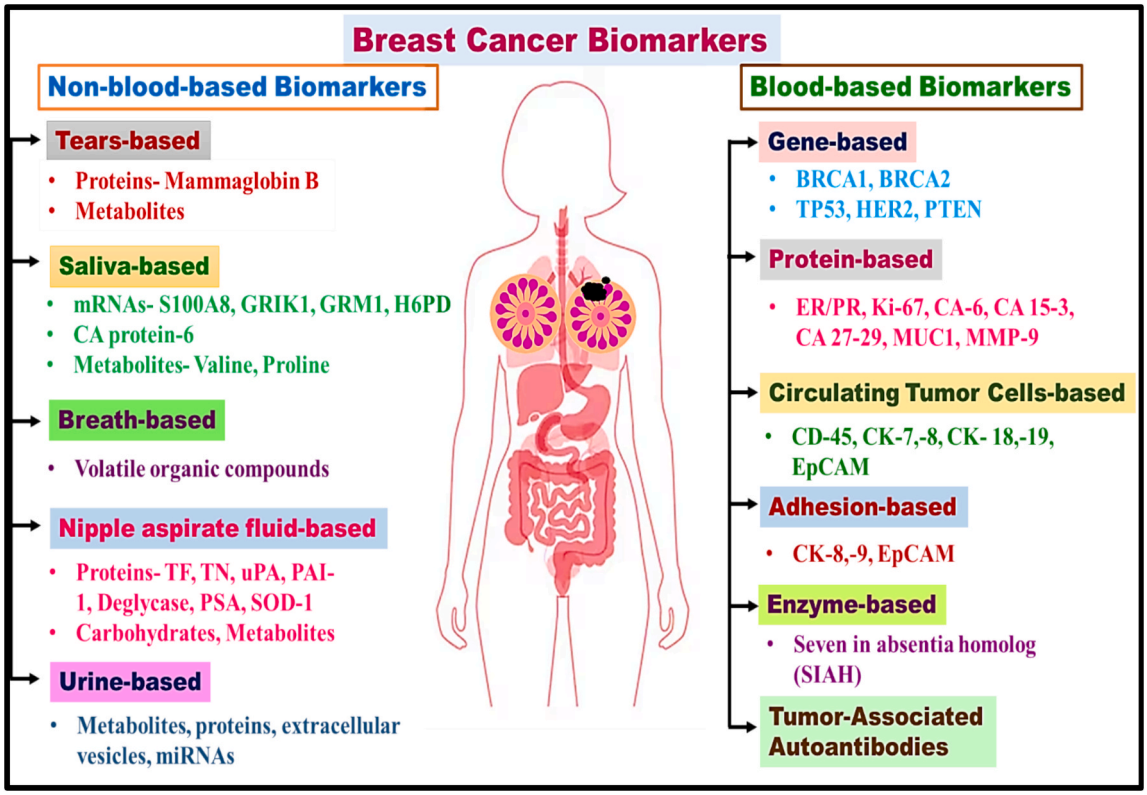

Fig. 2c. Diagram illustrating important biomarkers for breast cancer diagnosis. (Abbreviations of biomarkers: S100A8- S100 Calcium Binding Protein A8, GRIK1- glutamate receptor ionotropic, kainate 1, GRM1- glutamate metabotropic receptor 1, H6PD- hexose-6-phosphate dehydrogenase , CA protein-6- carbonic anhydrase 6, TF- Thomsen-Friedenreich antigen, TN- Tn antigen, uPA- urokinase – type plasminogen activator, PAI-1- Plasminogen Activator Inhibitor-1, PSA- Prostate-specific antigen, SOD-1- Superoxide Dismutase-1, *BRCA*- BReast CAncer gene, *TP53*- Tumor protein 53, *HER2*- human epidermal growth factor receptor 2, *PTEN*- phosphatase and tensin homolog, ER/PR- Estrogen Receptor/Progesterone Receptor protein, Ki-67- Marker of Proliferation Ki-67, CA- cancer antigens, MUC1- Mucin-1, MMP-9- Matrix metalloproteinase-9, CD-45- Cluster of Differentiation 45, CK- Cytokeratin, EpCAM- epithelial cell adhesion molecule).

Table 2
Various genes, associated signalling pathways and their role in breast cancer.

Genes	Function	Pathways associated	Role in breast cancer	References
BRCA1 (BReast CAncer gene 1) and ***BRCA2*** (BReast CAncer gene 2)	They are tumor suppressor genes involved in DNA repair via homologous recombination.	DNA damage repair pathway	They are commonly associated with hereditary breast cancer. Mutations in these genes significantly increase the risk of breast cancer and lead to impaired DNA repair, allowing genetic errors to accumulate, and driving cancer development.	[27]
TP53 (tumor protein p53)	*P53* is a tumor suppressor gene that regulates cell cycle, DNA repair, and apoptosis.	p53 signalling pathway	Loss of *p53* function leads to uncontrolled cell growth and resistance to apoptosis.	[28,29]
PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha)	It is an oncogene that encodes the p110α subunit of PI3K, and is involved in cell growth and survival.	PI3K/AKT/mTOR pathway	Mutations in *PIK3CA* lead to hyperactivation of the PI3K pathway, promoting cell proliferation and survival.	[30]
HER2 (Human Epidermal Growth Factor Receptor 2) or ***ERBB2*** (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2)	*HER2* is an oncogene that encodes a receptor tyrosine kinase involved in cell growth and differentiation.	HER2/ERBB signalling pathway	*HER2* overexpression leads to uncontrolled cell division and growth.	[31,32]
PTEN (Phosphatase and tensin homolog)	*PTEN* is a tumor suppressor gene encoding phosphatase	PI3K/AKT/mTOR pathway	Loss or mutation of *PTEN* leads to unregulated activation of the PI3K/AKT pathway.	[33]
CDH1 (E-cadherin)	*CDH1* is a tumor suppressor gene encoding E-cadherin, involved in cell adhesion.	Cell adhesion and Wnt signaling pathway	Loss of E-cadherin disrupts cell adhesion, leading to increased invasion and metastasis.	[34]
RB1 (Retinoblastoma 1)	It is a tumor suppressor gene and blocks cell cycle progression by inhibiting E2F (early region 2 binding factor) transcription factors.	Cell cycle pathway	Mutations or loss of *RB*1 function can lead to unchecked cell proliferation by unregulated G1/S transition.	[35]
GATA3 (GATA Binding Protein 3)	It is a transcription factor involved in the differentiation and function of luminal cells in the breast	Estrogen receptor signalling pathway	*GATA3* is involved in maintaining the differentiation of luminal cells and its mutation can contribute to tumorigenesis.	[36]
ESR1 (Estrogen Receptor 1)	ESR1 is a nuclear receptor mediating the effects of estrogen in cell growth and differentiation.	Estrogen receptor signalling pathway	Mutations in *ESR1* can lead to constitutive activation of the estrogen receptor, promoting the growth of hormone receptor-positive breast cancers.	[37,38]
MYC (myelocytomatosis oncogene)	It is a proto-oncogene linked with various cancers	MYC signalling pathway.	Amplification or overexpression of *MYC* drives uncontrolled cell growth, metabolism, and proliferation.	[39]
AKT1 (AKT Serine/Threonine Kinase 1)	It is an oncogene involved in cell survival, growth, and metabolism	PI3K/AKT/mTOR pathway	Mutations in *AKT1* can lead to constitutive activation of the PI3K/AKT/mTOR pathway, promoting tumorigenesis	[40]
EGFR (Epidermal growth factor receptor)	*EGFR* is an oncogene involved in cell growth and differentiation	EGFR signalling pathway	Overexpression or mutation of *EGFR* is associated with aggressive forms of breast cancer	[41]
STK11 (Serine/Threonine Kinase 11)	*STK11* is a tumor suppressor gene that encodes a protein kinase involved in regulating cell metabolism, cell polarity, and the cell cycle.	AMPK (AMP-activated protein kinase) Pathway; mTOR (mammalian target of rapamycin) Pathway	Mutation in *STK11* results in increased cell proliferation, increased metabolism, and loss of cell polarity.	[42]

Pathogenicity of breast cancer is influenced by multiple factors ranging from intrinsic genetic mutations, hormonal imbalances, and metabolic influences to extrinsic environmental factors. Mutations in important tumor suppressor genes like *TP53, STK11, RB1, PTEN, BRCA1, and BRCA2* cause irregulation of cell cycle, loss of cell integrity, and impaired ability to repair DNA, resulting in unchecked cell division and tumor growth [43]. Oncogenes further increase this uncontrolled growth and promotes dysregulation of the important signalling pathways [44]. Some of the major pathways involved in the breast cancer progression are depicted in Fig. 3a.

Breast cancer cells have altered metabolic phenotypes depending on their subtypes and the site of metastasis. Factors such as overexpression of *MYC, PIK3CA*, and mutation in *p53* along with hypoxia, oxidative stress, and acidosis results in the changed metabolic phenotypes. A breast cancer cell generally has enhanced metabolism of glucose, lipids, and amino acids that has been achieved by metabolic reprogramming. These tumor cell utilize glycolysis and pentose phosphate pathway to produce ATP and NADPH respectively [45]. An explanatory description of the metabolic pathways in breast cancer cells has been shown in Fig. 3b.

3. Breast cancer detection

Early detection of breast cancer is necessary for improving survival rates of the patients and designing their treatment strategies. There have been noteworthy developments in the breast cancer diagnostics and various detection strategies have been incorporated for the early and precise diagnosis. Detection methods ranging from conventional methods to advanced methods could be categorized into imaging techniques, molecular diagnostics, liquid biopsy, and biosensing techniques as depicted in Fig. 4a. In this review some of the conventional detection strategies for breast cancer and new-age biosensing techniques with special emphasis on CRISPR-based biosensors will be discussed.

3.1. Conventional techniques for the detection of breast cancer

The detection of breast cancer is essential for decreasing the associated morbidity, designing the requisite treatment strategy, and improving the survival outcomes of the patient. Conventional techniques like mammography, ultrasound, and Magnetic Resonance Imaging (MRI) have remained critical in the screening and diagnosis due to their established efficacy and accessibility. Different conventional breast cancer diagnostic techniques along with their advantages and disadvantages are described in Table 3. These methods are widely used in the

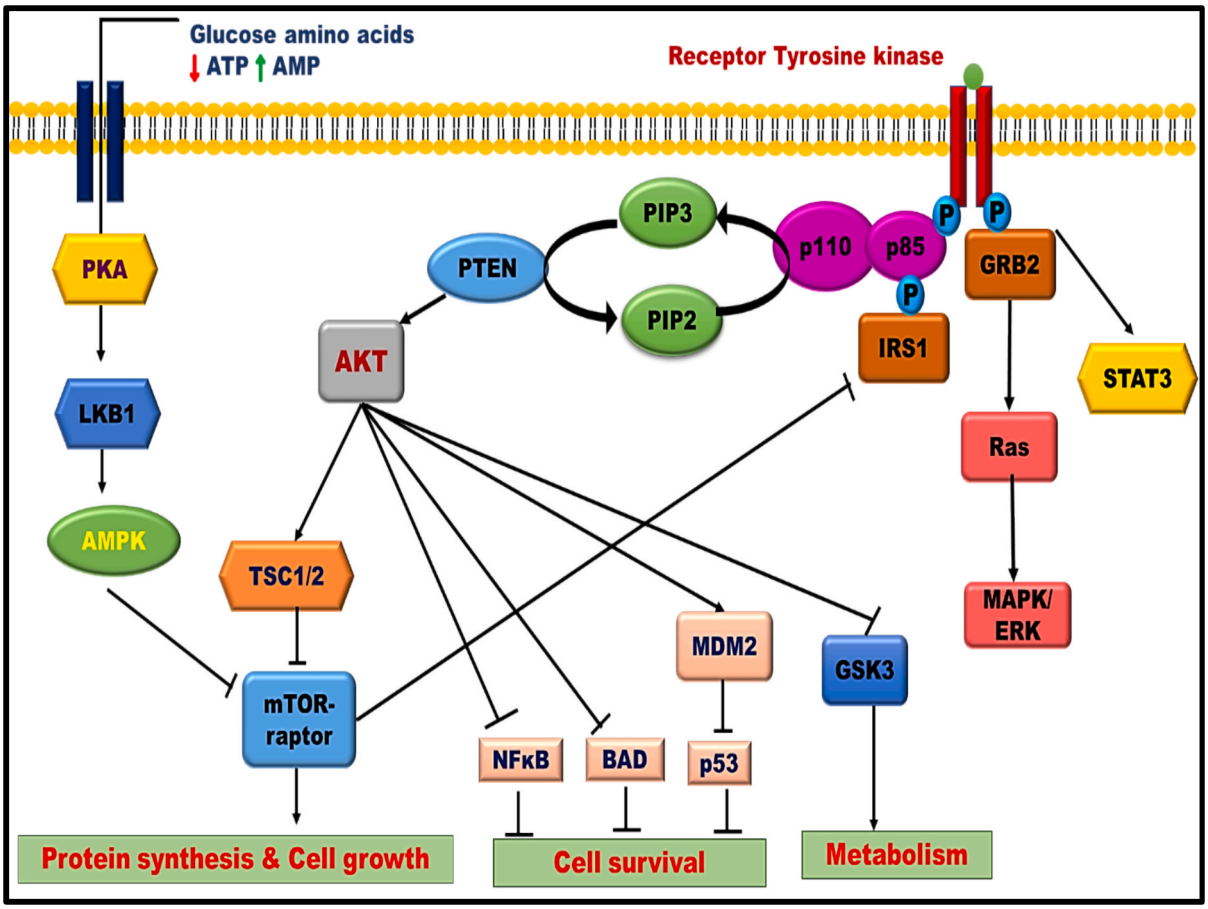

Fig. 3a. Schematic representation of various pathways involved in breast cancer progression. (Abbreviations of genes: *PKA*- Protein kinase A, *LKB1*- STK11 serine/threonine kinase 11, *AMPK*- AMP-activated protein kinase, TSC1/2- Tuberous sclerosis complex protein 1, mTOR-raptor- mechanistic target of rapamycin, *PTEN*- phosphatase and tensin homolog, NFκB- Nuclear factor kappa-light-chain-enhancer of activated B cells, *BAD- Bcl-2* agonist of cell death, *MDM2*- murine double minute 2, PIP- Phosphatidylinositol 4,5-bisphosphate, *GSK3*- glycogen synthase kinase 3, *IRS1*- Insulin Receptor Substrate 1, *GRB2*- growth factor receptor bound protein 2, *STAT3*- signal transducer and activator of transcription 3, *MAPK/ERK*- mitogen-activated protein kinase/extracellular signal-regulated kinase).

routine examination to detect small tumors, distinguishing between benign and malignant stages, and assessing the extent of metastasis [46]. Despite the advancements in breast cancer research and development of modern molecular imaging techniques, they face significant drawbacks necessitating the need of development of new technologies. Major challenge is posed while dealing with dense breast tissues where there is variability in accuracy. Additional limitations include low sensitivity, low specificity, false positives, and negatives etc. when dealing with dense breast tissues [47].

3.2. Biosensing techniques for the detection of breast cancer

Traditional approaches for the detection of breast cancer faces numerous short comings [60]. They often detect only structural abnormalities, which only develops after the progression of cancer. Hence, new and advanced technologies are needed to enhance the sensitivity, specificity which leads to less invasive and earlier detection of breast cancer. The limitations of conventional strategies and progression of research has driven the fabrication of biosensors for the detection of breast cancer.

Biosensors are the analytical devices which could precisely identify the target analyte from a complex mixture [61]. Fig. 4b represent the principle and procedure of working of a biosensor. These biosensors offer propitious solution to these limitations by offering early detection of breast cancer at the molecular level, often before tumors get visible through imaging. They detect specific breast cancer biomarkers present in blood, saliva, or urine hence, improving treatment outcomes [62]. The biosensors can be designed to be highly specific, which will further decrease the risk of false positives and will provide more reliable diagnostic information. Biosensing technologies have revolutionized breast cancer diagnostics by reducing the radiation exposure, and offering faster, cost-effective, accurate, and accessible detection methods [5].

3.3. Role of nanomaterials in biosensors for enhanced breast cancer detection

Recent progress in nanotechnology has led to a significant increase in the advancement of biosensing devices for breast cancer detection [63]. Integrating nanomaterials with the biosensor can address the challenges posed by conventional detection strategies. The unique properties of nanomaterials, such as their high surface area to volume ratio, biocompatibility, stability, conductivity, and tunable physical and chemical characteristics, make them ideal for use in biosensor fabrication.

Nanomaterials like gold nanoparticles [64,65], silver nanoparticles [66], carbon based nanostructures [67,68], and quantum dots [69,70] can be engineered to interact with specific breast cancer biomarkers. These nanomaterials allow minute detection of breast cancer related biomarkers by amplifying the signal generation and enhancing sensitivity. The versatility of nanomaterials allows for the creation of miniaturized and multiplexed biosensors that could detect multiple biomarkers simultaneously with high precision [71]. Nanomaterial-enhanced biosensors offer a transformative approach to breast cancer diagnostics by providing reliable, cost-effective, and accessible point of

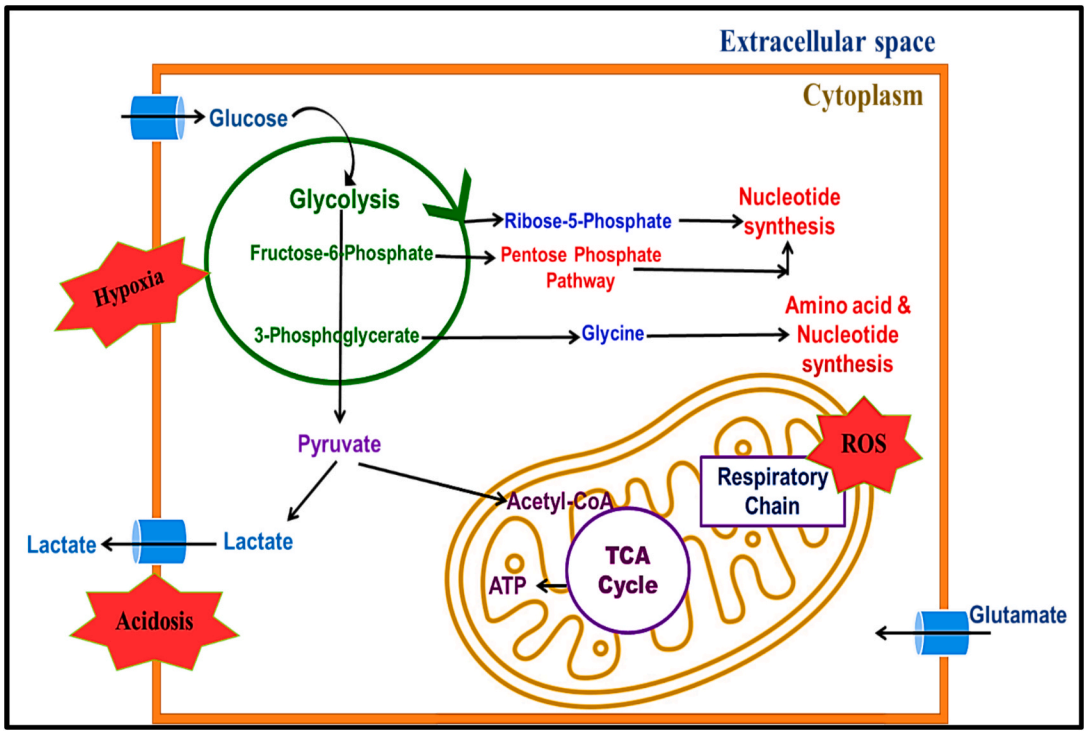

Fig. 3b. Schematic representation of metabolic pathways involved in breast cancer progression.

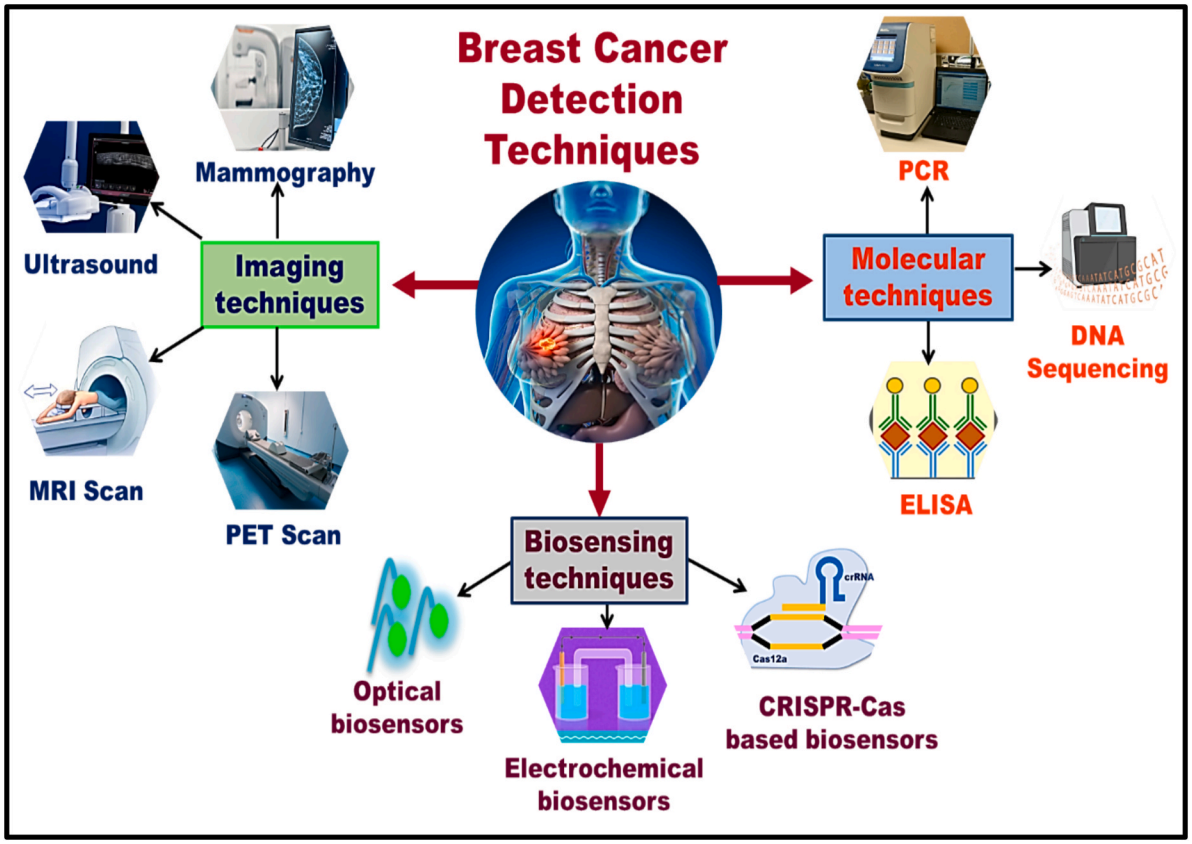

Fig. 4a. Diagrammatic illustration of various breast cancer detection techniques.

Table 3
Comparative depiction of various conventional techniques for the detection of breast cancer.

Detection technique	Description	Advantages	Limitations	References
Mammography	It is an X-ray imaging technique specifically used for diagnosing breast tissue.	• It can be used for imaging bone, soft tissue and blood vessels at the same time. • It is easily accessible and mostly used in routine examination. • Mammography is relatively less expensive.	• This technique involve exposure to ionizing radiations. • Mammograms can sometimes yield false positive results. • The procedure can cause discomfort as require breast compression for clear imaging. • Mammography can have less sensitive detection with increased tissue density.	[46]
Ultrasound	It involves the use of high frequency sound waves to create images of the breast tissue.	• Ultrasound doesn't involve exposure to harmful radiations, hence, considered safe. • It could be used to detect lumps found in mammography. • It could distinguish between solid tumors and fluid filled cysts. • It is often used as a guide for needle biopsies for breast.	• It requires specialized technician. • It could also result in false positives. • It has low sensitivity and specificity. • Ultrasound images produced are of low resolution.	[47]
Magnetic Resonance Imaging (MRI)	MRI uses strong magnetic fields and radio waves to produce detailed images of the breast.	• It is highly sensitive and often used in conjunction with mammography. • It doesn't use ionizing radiations. • MRI provides detailed view of the breast and surrounding tissues, to assess extent of cancer.	• MRI is more expensive than other imaging techniques. • It can lead to false positives. • It requires specialized equipment and expertise.	[48]
Digital Breast Tomosynthesis (DBT) (3D Mammography)	It is the advanced version of mammography that creates 3D image of the breast by taking multiple X-ray images from different angles.	• DBT is considered more sensitive in detecting cancer than conventional mammography. • It has lower rate of false positives. • It provides clear view of the breast overlapping tissues for easy identification of tumors.	• DBT involves higher exposure to the radiations. • DBT is not widely available. • It is more expensive than conventional mammography.	[49]
Breast Biopsy	Biopsy involves the removal of a small sample of breast tissue for examination under microscope.	• Biopsy is a confirm way to diagnose breast cancer.	• Procedure of biopsy is invasive and can cause discomfort to patient.	[50]
Molecular Breast Imaging (MBI)	MBI uses certain radioactive tracer and a special camera to detect cancer cells in the breast.	• It is highly effective in detecting tumors within dense breast tissue. • It is a functional imaging technique.	• It involves high exposure to the radiations. • It is not available widely. • The procedure is expensive and not used in routine screening.	[51,52]
Positron Emission Tomography (PET) Scan	PET is the nuclear imaging technique that is used to detect areas of high metabolic activity, characteristic of cancer cells by using specific amount of radioactive sugar tracer.	• PET scans provide a comprehensive view of the entire body hence, useful in detection of metastasis. • It could also identify potential cancer cells which had not yet formed a tumor.	• PET scans are highly expensive. • There is a significant amount of exposure to radiations involved during PET scan. • It could also result in false positive results.	[53,54]
Clinical Breast Examination (CBE)	CBE involves manual inspection of breast by a healthcare professional to detect any lumps or abnormalities.	• It is a simple procedure and requires no specialized equipment. • It is of low cost and can detect lumps that patients might not notice.	• CBE has very less sensitivity than any other method. • It is not always accurate. • It is not alone sufficient in diagnosing breast cancer.	[55,56]
Thermography	It is a thermal imaging technique used to detect heat patterns and blood flow in the breast tissue.	• It does not use any ionizing radiations. • It is a non-invasive procedure.	• It has low sensitivity and variations in results. • It is not recommended as a stand-alone screening tool.	[57]
Immunohistochemistry (IHC)	IHC is used for visualization of specific antigens using antigen–antibody interactions in tissue sections.	• It is a standardized procedure and widely used in labs. • It provides visual evidence of protein expression profiles and localization.	• The sample preparation, staining, and analysis is time consuming. • There are chances of cross-reactivity, leading to false positive results. • IHC is not fully quantitative and the results and analysis are subjective.	[58]
Fluorescence In Situ Hybridization (FISH)	It is a cytogenetic technique that is used to detect genetic abnormalities like gene amplifications, deletions, and translocations, with the help of fluorescent probes.	• FISH can detect cancer associated genetic abnormalities with high sensitivity. • It allows for direct *in situ* visualization. • It can also allow quantitative estimation of gene copy number.	• The sample preparation is complex and labour-intensive. • It is not suitable for multiplex detection. • FISH is generally expensive and resource-intensive.	[59]

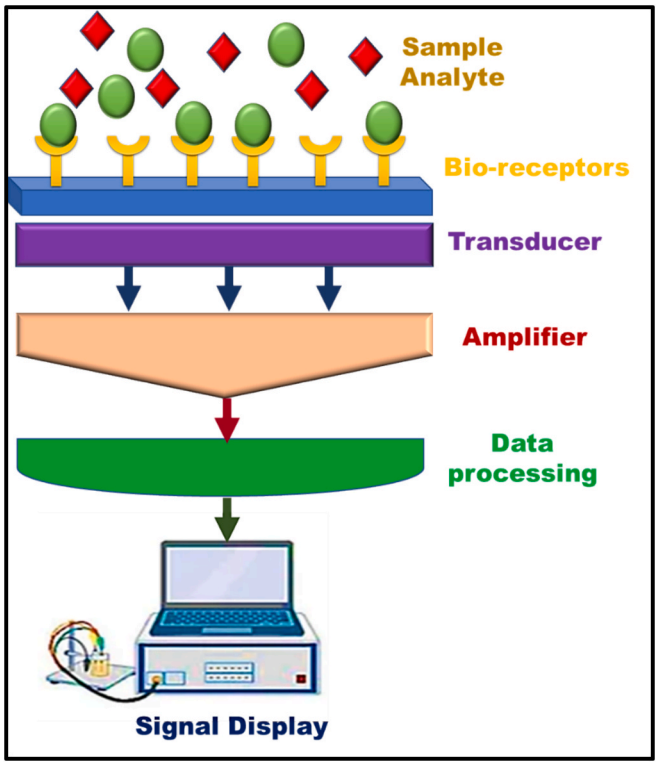

Fig. 4b. Schematic representation of principle and working of a biosensor.

care solution. Fig. 4c explains the significance of using nanomaterials in biosensors for detecting breast cancer.

3.4. *Clustered regularly interspaced short palindromic repeats (CRISPR) technology in biosensors*

CRISPR technology has revolutionized the field of genetic engineering and molecular biology. It was originally discovered in the bacteria as a part of their immune system offering protection against invading viruses [72]. CRISPR-Cas (CRISPR-associated proteins) acts as a molecular scissor which cut DNA at specific locations within the genome.

3.4.1. *Mechanism of CRISPR technology*

The CRISPR-Cas system is composed of two important components, guide RNA (gRNA) to guide Cas enzyme to the target location, and Cas enzyme, acting as the nuclease to cut DNA at the specific location [73]. The mechanism of cleavage by CRISPR involves, incorporation of a viral DNA fragment in the CRISPR locus in the form of a new spacer. The CRISPR locus after transcription produces a precursor RNA which get processed into CRISPR RNA (crRNA). This crRNA either get paired with trans-activating CRISPR RNA (tracrRNA) or as a single gRNA, guides Cas protein to bind with its complementary DNA/RNA target hence, resulting in cleavage of the target [74]. The working mechanism of CRISPR technology employed in biosensing strategies is explained through Fig. 4d.

CRISPR systems is categorized in two classes namely, class 1 and class 2 systems. Class 1 systems use multi-protein complexes for recognizing the target and its cleavage. It is further divided in types and subtypes of type I (Cas 3), type III (Cas 10), and type IV (Csf 1). Class 2 systems are widely used in biotechnology owing to their simplicity and effectiveness. They use a single, multifunctional Cas protein and includes type II (Cas 9), type V (Cas 12 & Cas 14), and type VI (Cas 13).

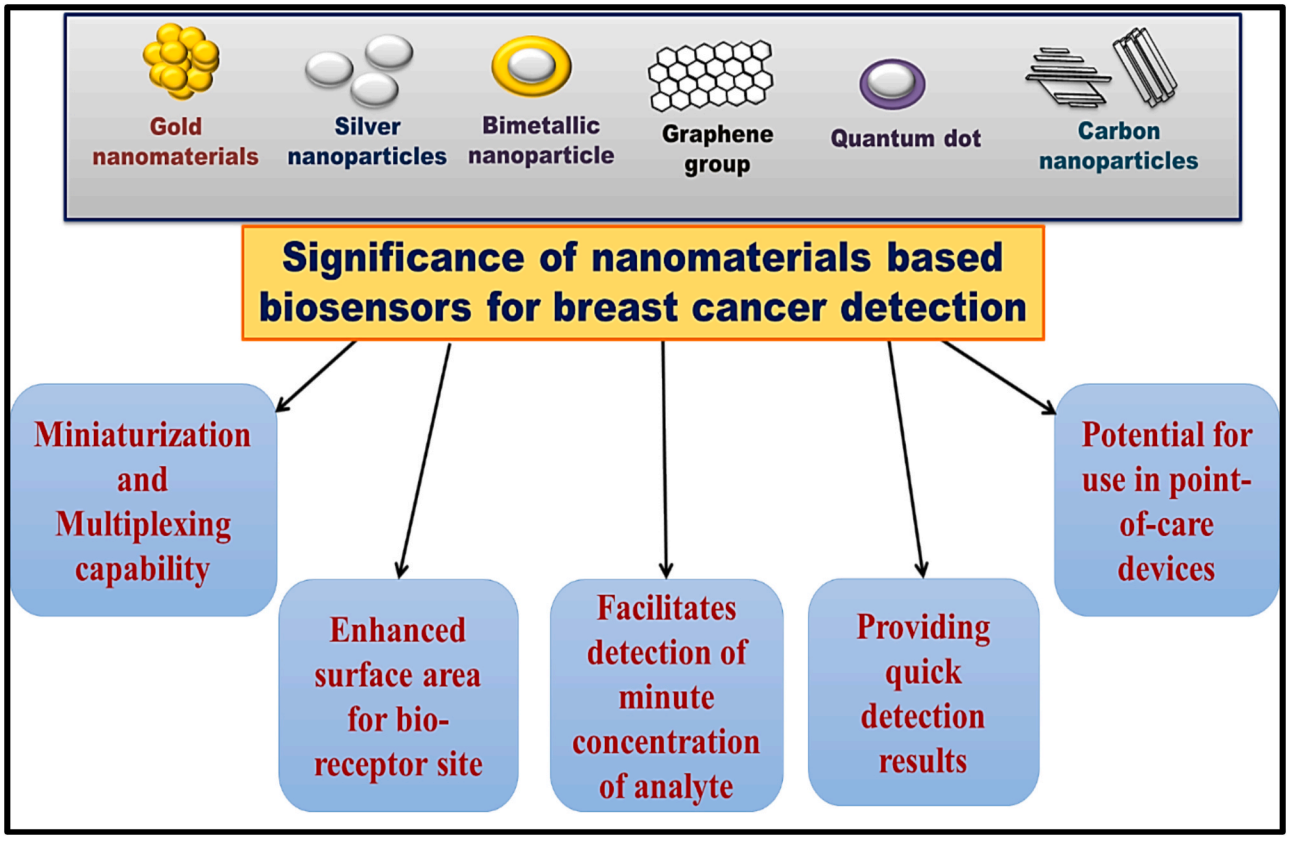

Fig. 4c. Diagrammatic illustration of nanomaterials and their significance in the fabrication of biosensors for breast cancer detection.

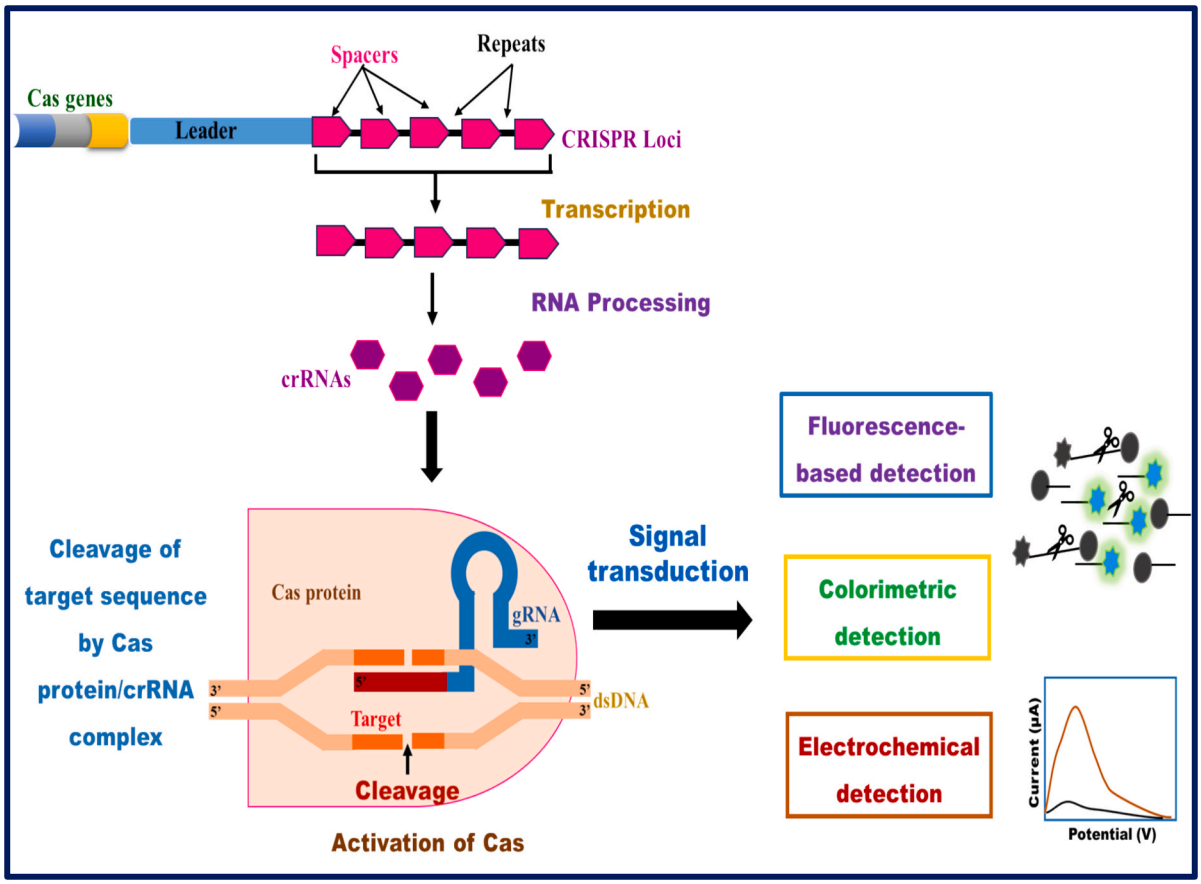

Fig. 4d. Figure representing working mechanism of CRISPR-Cas technology in biosensors.

Cas9 is a dsDNA endonuclease guided by RNA and is used generally for genome editing. Cas 14 is a smaller variant and have high specificity for ssDNA. While Cas12 and Cas 13 cleaves dsDNA and RNA respectively and exhibits collateral cleavage activity [6]. This capability of CRISPR-Cas system to precisely target and identify specific DNA or RNA sequence is being utilized to develop biosensing devices for breast cancer diagnostics [75].The guide RNA designed to bind with the complementary target sequence associated with the breast cancer biomarkers causes activation of the Cas enzyme. The activated Cas enzyme causes collateral cleavage of the surrounding nucleic acids which is a significant step for the amplification of signal in the biosensor [76]. This innovative technology offers numerous advantages over conventional detection methods as well as over traditional biosensors, including heightened sensitivity, specificity, and the potential for rapid, point-of-care, non-invasive breast cancer diagnostics.

3.4.2. Advantages of CRISPR-Cas based biosensors for breast cancer detection

CRISPR-based biosensors are ideal for early stage detection of breast cancer biomarkers as they can detect their extremely low levels with high sensitivity [6]. This sensitivity is crucial for identifying cancer at molecular level in the early stage. They offer more accurate diagnostic results with low false positives owing to the ability of highly specific detection. CRISPR-based biosensors can deliver results in short period of time, often within few minutes to hours [77]. This quick and rapid detection is important in low resource clinical settings. Owing to the portability and simplicity of CRISPR-based biosensors, they are particularly valuable for point-of-care diagnostics. They are designed in such a way to reduce the need of more invasive biopsy by analyzing non-invasive bodily fluids such as blood, urine, or saliva. This further enhances the patient comfort and screening [78]. CRISPR-Cas biosensors could also be used to monitor treatment response by analyzing the levels of specific biomarkers during and after treatment [79]. With the advancement of technology, CRISPR-based biosensors are promising to revolutionize breast cancer detection, offering more precise, accessible, and personalized diagnostic tools that could significantly improve treatment strategies as well as patient outcomes.

4. Applications of CRISPR-Cas based biosensing techniques for breast cancer detection

CRISPR-Cas based biosensors have achieved a considerable attention in the field of breast cancer diagnostics [80]. CRISPR-Cas system is integrated in the biosensing platform to recognize specific DNA/RNA sequences associated with breast cancer. These biosensors have a huge potential in the detection of breast cancer biomarkers and providing a highly sensitive and specific analysis [6]. Upon recognition of the target, the Cas protein cleave the DNA/RNA initiating a signal such as fluorescence, colorimetric, or electrochemical changes. In the following sections, we explain applications of CRISPR based distinct biosensing devices for the breast cancer biomarkers detection:

4.1. CRISPR-based electrochemical biosensors for the breast cancer detection

Electrochemical biosensors are the analytical devices which combines the biological recognition element with an electrochemical transducer to translate a biological interaction into an electrical signal [81]. They are highly powerful tools for the detection of breast cancer biomarkers with great sensitivity and precision [82]. The integration of CRISPR technology with them further alleviates the detection potential and specificity [83].

Sha et al. (2024) fabricated an electrochemical CRISPR-based biosensor for the diagnosis of breast cancer. The biomarker targeted by them was circulating tumor cell (CTC) with the overexpressed levels of folate receptor [84]. Folate receptor is a cell surface receptor glycoprotein overexpressed on breast cancer cells. The working mechanism of biosensor involved use of folate-functionalized magnetic beads to capture folate receptor-positive CTCs. The cell membranes then underwent mild reduction and exposed free-sulfhydryl groups coupled with maleimide-DNA. This further resulted in the activation of collateral cleavage activity of CRISPR/Cas12a system and cleaved long DNA chains modified with methylene blue in small fragments. These fragments were then captured through host–guest recognition on a graphite electrode, generating electrochemical signal in proportion with the number of CTCs. This highly sensitive biosensor was able to detect CTCs from the peripheral blood with a detection limit of 2 cells/mL. The biosensor also achieved high specificity by the use of folate-functionalized magnetic beads hence, low false positives from normal cells. The combination of mild reduction-activated surface labeling and CRISPR/Cas12a *trans*-cleavage activity resulted in a strong electrochemical signal, with enhanced detection sensitivity. The fabricated biosensor had also shown excellent stability in complex environments such as, peripheral blood, making it reliable for clinical applications in early stage breast cancer diagnosis. Along with the mentioned advantages, this biosensor had some limitations in the form of complex fabrication steps, challenges in sample preparation, and limited applicability to other types of breast cancer biomarkers.

Another CRISPR-Cas12a based biosensor was developed by Hu et al. (2024), combining electrochemical sensing with aptamer technology for the detection of breast cancer derived exosomes [85]. In this study aptamer specific to epithelial cell adhesion molecule (EpCAM) is conjugated to magnetic nanoparticles (MNPs) via a biotin-streptavidin interaction. In the presence of breast cancer-derived exosomes, the aptamer on binding with the exosome surface proteins, released a partially complementary DNA strand (P1). The released P1 activated CRISPR/Cas12a by interacting with crRNA, which resulted in cleavage of a methylene blue-labelled single-stranded DNA reporter (P2) attached to gold electrode. The cleavage of P2 altered the electrochemical signal, allowing for the detection of exosomes as depicted in Fig. 5a. The biosensor was tested on clinical blood samples to differentiate between healthy individuals and breast cancer patients. The limit of detection obtained was 280 particles/mL. The developed biosensor was highly sensitive, specific, and non-invasive diagnostic tool that used liquid biopsy from blood samples, avoided need for tissue biopsies. In this electrochemical biosensor, background noise although minimized, could still interfere with detection. However, while the biosensor demonstrates impressive performance in initial tests, further clinical validation is essential to establish its reliability and usability in broader medical applications. Overall, this work represents a significant advancement in the development of biosensors for cancer detection, providing a promising platform for future diagnostic tools.

A study by Dong et al. (2024) presented an innovative CRISPR/Cas12a-mediated ratiometric dual-signal electrochemical biosensor for the ultrasensitive and reliable detection of circulating tumor DNA (ctDNA), specifically targeted for the *KRAS* gene mutation. The biosensor detected ctDNA by leveraging the exponential amplification reaction (EXPAR) and generated activators for the CRISPR-Cas12a system [86]. The ssDNA-P sequence, complementary to the methylene-blue modified ssDNA biogate (ssDNA-MB) was then cleaved by Cas12a. This cleavage prevented the separation of ssDNA-MB from the UiO-66-NH_2 surface which hindered the release of signal molecules i.e. methylene blue and ferrocene. If the target ctDNA was absent, the ssDNA-MB biogate remained opened it caused leakage of ferrocene molecules and signal generation. In this study detection principle relied on a ratiometric strategy where CRISPR/Cas12a acted as a "connector," linking the presence of ctDNA to the ratio of signals from methylene blue and ferrocene hence, achieved significantly enhanced sensitivity compared to conventional single-signal methods. However, further development and clinical validation can make it a powerful tool in the early diagnosis of breast cancer.

4.2. *CRISPR-based optical biosensing platforms for the detection of breast cancer*

Optical biosensors are the analytical devices that rely on the interaction between the target biomarker and a specific immobilized capture probe to produce a measurable change in the optical signal [87]. These

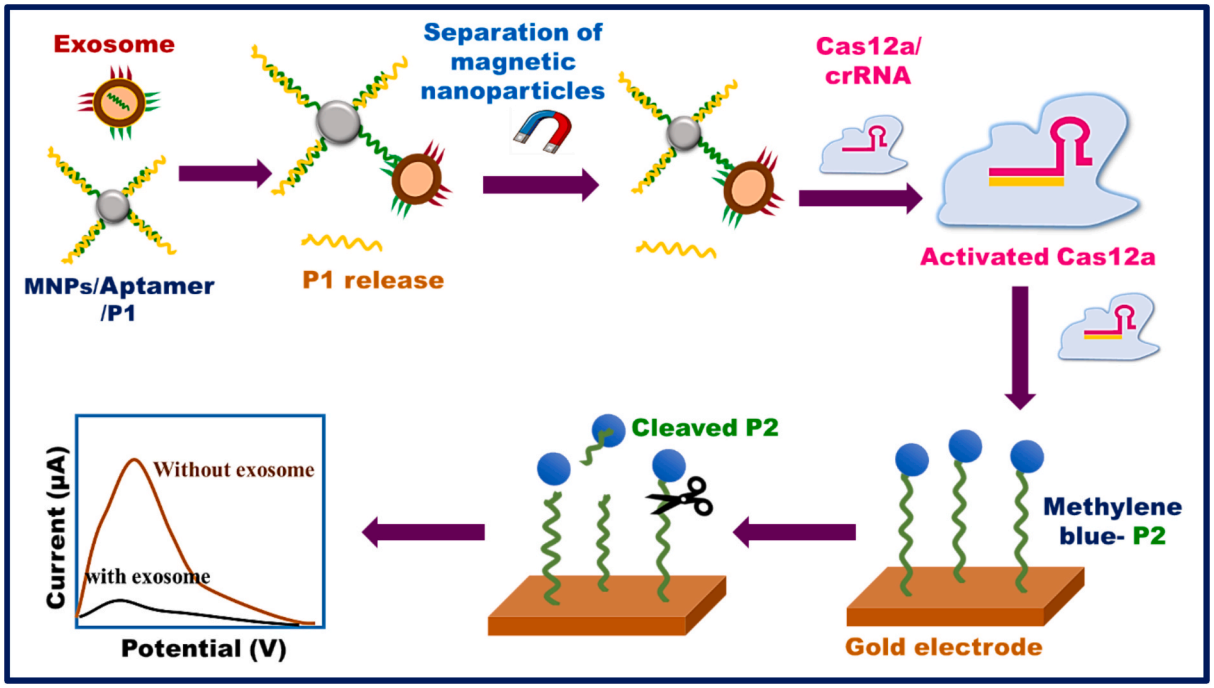

Fig. 5a. Schematic illustration of detection of breast cancer derived exosomes by using a CRISPR-Cas12a based electrochemical biosensor.

biosensors offer high sensitivity, specificity, and potential of real-time monitoring and detection of early-stage breast cancer.

4.2.1. *CRISPR-based fluorescence and colorimetric biosensors for breast cancer detection*

Peng et al. (2024) developed a biosensor to detect the overexpressed miRNA-21 in the breast cancer cells. They designed structural "Lock" DNA approach along with CRISPR-Cas 12a system [88]. The Lock-Cas12a platform utilized a "Lock" DNA that released crRNA from dependency on the target sequence. The target sequence was amplified by strand displacement amplification which further activated Cas12a. Cas12a then cleaved a fluorophore-quencher labelled reporter molecule that led to further amplification. The amplified signal was finally detected via fluorescence. The biosensor was able to detect minute concentration of miRNA-21 at even 28.8 aM concentration with great sensitivity. This biosensor was simple and straightforward with the detection process conducted at a constant temperature. This biosensor was adaptable for different targets as capable of detecting various miRNAs without needing to alter the crRNA spacer sequence. It was able to distinguish breast cancer cells from other types of cancer cells through intracellular imaging hence having application in in-vivo detection.

Exosomes have emerged as a potent biomarker for breast cancer diagnostics. These are the small extracellular vesicles that have the ability to carry proteins and nucleic acids which can determine state of parent cancer cells. Guan et al. (2024) in a study developed a CRISPR/Cas12a and aptamer-chemiluminescence-based analysis (CACBA) technique to detect the exosomes and their tumor specific surface proteins that were, EpCAM and MUC1. They exploited the specificity as well as amplification capabilities of the CRISPR/Cas12a system and the sensitivity of chemiluminescence detection [89]. The biosensor used magnetic beads that were functionalized with CD63 aptamers to capture exosomes. Captured exosomes were followed by the release of trigger sequences that activated the CRISPR/Cas12a system for fluorescence-based detection. This biosensor was able to achieve detection limit of 8.97×10^3 particles/μL for the total exosomes and 1.45×10^2 and 3.73×10^2 particles/μL for EpCAM and MUC1 positive exosomes, respectively. The fabricated biosensor was successful in distinguishing between breast cancer patients and healthy donors hence, offered a more precise, stable, and cost-effective tool for breast cancer diagnostics. This method can be adapted for the detection of other cancers by targeting different tumor-related exosomal proteins and can be used in real-life clinical settings.

In a different study Wang et al. (2024) targeted another potent breast cancer biomarker, flap endonuclease 1 (FEN1). FEN1 is a key enzyme involved in DNA replication and repair and its high expression are associated with aggressive and high grade, triple negative breast cancer phenotype. They designed a fluorescent biosensor based on CRISPR-Cas12a to detect FEN1 with high sensitivity [90]. The detection mechanism relied on the use of ligase detection reaction (LDR) for amplification of the target DNA sequence as described in Fig. 5b. It involved the ligation of two adjacent oligonucleotides in the presence of target DNA to form a continuous DNA strand. The amplified DNA product then turned on the collateral cleavage activity of Cas12a. Cas12a guided by crRNA on recognition of target DNA cleaved nearby fluorescent labelled ssDNA. The cleavage caused release of a detectable fluorescent signal indicative of the presence of target FEN1 enzyme. This fabricated biosensor was able to achieve a detection limit of 1.31×10^{-8} U. The standout feature of this biosensor was integration of LDR with CRISPR-Cas12a for single molecule counting of FEN1 with high sensitivity and specificity. The use of LDR has not only amplified the target DNA signal but also enhanced the sensitivity of CRISPR-Cas12a detection. The applicability of biosensor could also be extended beyond diagnostics in providing a powerful tool for drug discovery by screening FEN1 inhibitors hence further improving patient outcomes.

CD63 and vascular endothelial growth factor (VEGF) are co-expressed on breast cancer-derived small extracellular vesicles (tEVs). Tan et al. (2024) selected CD63 and VEGF as biomarkers and developed a CRISPR-based biosensor for the detection of breast cancer. The biosensor used dual aptamers targeted at CD63 as well as VEGF, if they both were present on the same tEV, the aptamers hybridized in close proximity [91]. This hybridization resulted in the formation of a DNA complex that acted as a signal generator. This signal triggered the "DNA gate-based exponential amplification CRISPR-Cas (DGEAC) system", driven by the *trans*-cleavage activity of Cas12a, to produce a strong fluorescent signal. The biosensor was able to achieve ultrasensitive detection of tEVs with a limit of detection (LOD) of 1.02×10^3 particles/mL in a linear range of 1.75×10^3 to 3.5×10^8 particles/mL. The dual-aptamer-based AND Gate design of the system provided an accurate and specific method for detection by requiring both biomarkers to trigger the signal. The biosensor demonstrated the potential for non-invasive of

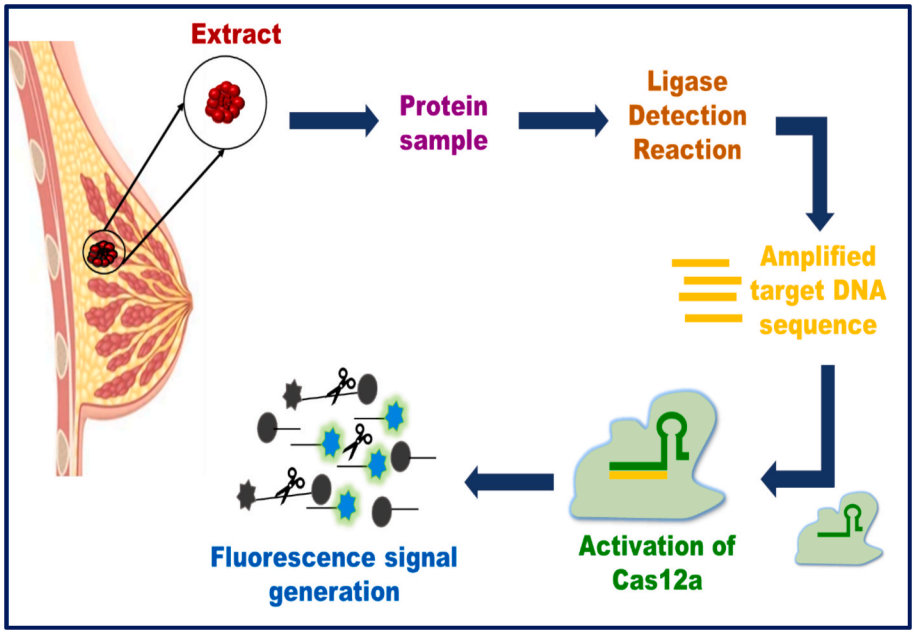

Fig. 5b. Diagrammatic representation of FEN1 detection by using a CRISPR-Cas12a and ligase detection reaction-based fluorescence biosensor.

breast cancer-derived tEVs detection, especially in differentiating cancer stages.

There are a numerous research studies undergoing to establish a less invasive diagnostic strategy for the breast cancer. One of such approach is liquid biopsy that target tumor-derived components in the bodily fluids such as circulating cell-free DNA (cfDNA) [92]. One such attempt was done by Zhao et al. (2024) by designing a dual mode (colorimetry and fluorescence) biosensor for the detection of cfDNA. The detection mechanism involved formation of Cas12a-cfDNA complex and activation of *trans*-cleavage activity [93]. Duplex-specific nuclease (DSN) further enhanced the signal by selectively cleaving DNA/RNA hybrids hence, recycling the target sequences and repeated signal generation. The biosensor was highly sensitive and specific and provided both fluorescent and colorimetric outputs. The cleavage of reporter molecule tagged with fluorophore and quencher resulted in fluorescence generation and aggregation of gold nanoparticles (AuNPs) induced by cleaved DNA sequences resulted in colorimetric detection. This CRISPR-Cas12a-based dual-mode biosensor provides a sensitive, selective, accurate, and reliable method for detecting cfDNA, particularly for the BRCA-1 gene associated with breast cancer. It also doesn't involve any nucleic acid amplification steps hence, avoiding associated potential false positives. The dual-mode output offers significant improvements in analytical precision and enhances the potential for use in point-of-care settings.

CRISPR-Cas system represents a significant advancement in the field of breast cancer diagnostics by offering a powerful tool for multiplexed detection. Tan et al. (2024) leveraged the multiplexed detection capability of CRISPR-Cas system in designing a fluorescence based biosensing device for the subsequent detection of circular RNA- circROBO1 and gene- *BRCA1* breast cancer biomarkers. circROBO1 is a circular RNA associated with poor prognosis in breast cancer and *BRCA1* is a well-known tumor suppressor gene associated with high-risk breast cancer. The biosensor utilized the collateral-cleavage activity of CRISPR-Cas13a (RNA-targeting enzyme) and CRISPR-Cas12a (DNA-targeting enzyme) for the concurrent recognition of circROBO1 and *BRCA1* respectively [94]. Upon recognition of their respective targets, circROBO1 and BRCA1, the CRISPR complexes cleave RNA and DNA reporters, resulting in the recovery of fluorescence signals FAM (6-carboxyfluorescein) and ROX (6-carboxy-xrhodamine) respectively. This fabricated biosensor offered a non-invasive and rapid alternative to traditional methods, with detection limits of 0.013 pM and 0.26 pM for circROBO1 and BRCA1 respectively. However, while this biosensor holds a great potential for clinical applications, its complexity, cost, and the potential impact of sample matrix effects must be tackled before widespread adoption. Despite these challenges, the platform's potential for multiplexed detection and point-of-care integration could make it a powerful tool in personalized medicine and improving patient outcomes.

In another study, Zhao et al. (2022) used metal-oxide framework (MOF) and CRISPR-Cas12a to design a bio-barcode biosensor for the detection of miRNA-21. The biosensor used DNA-functionalized zirconium-based MOFs as the bio-barcode system for conversion and amplification of miRNA targets [95]. These amplified multiple activators were then detected by CRISPR-Cas12a system which triggered a strong fluorescence signal for miRNA detection. The fabricated biosensor achieved attomolar sensitivity due to the dual amplification provided by MOFs and CRISPR-Cas12a. The versatility of this detection method allows it to be adapted for various miRNAs, broadening its potential impact in molecular diagnostics.

4.2.2. Liposome-mediated optical biosensors for breast cancer detection

Liposomes are tiny, spherical, lipid-soluble vesicles, consisting of one or more phospholipid bilayers and an interior aqueous core. They are often used as nanocarriers for various applications [96]. Zhou et al. (2024) in a study utilized cationic liposomes and hybridized them with exosomes to construct a CRISPR-Cas13a-based biosensor for the detection of miR-1246. miR-1246 is exosomal micro-RNA associated with breast cancer and is considered as a potent biomarker. The study involved creation of hybrid between exosomes and cationic liposomes loaded with CRISPR-Cas13a, crRNA, and RNA reporter probes through electrostatic interactions [97]. In the presence of exosomal miR1246, CRISPR-Cas13a got activated and cleaved RNA reporter probes within the liposomes. This resulted in generation of a fluorescence signal in proportion with the concentration of target miRNA. The sample investigated in this study included exosomes from breast cancer cell line MCF-7 and serum samples from breast cancer patients. The detection limit was obtained to be as low as 3×10^2 particles/mL. the biosensor was highly specific and was even able to differentiate between early and advanced stages of breast cancer. This biosensor is unique and different from other physical biosensors as it allowed for in-situ detection of miRNAs within the intact exosome structure, enhancing the detection accuracy and preserving the biological context. It is non-invasive and could be potentially used for monitoring disease progression. However, the construction of liposome-exosome hybrids would be complex and specialized equipment would be required for fluorescence signal detection.

Similarly, Zhang et al. (2023) in a different study fabricated a liposome-based biosensor for the detection of breast cancer associated exosomal miRNA-21. The biosensor utilized liposome-mediated membrane fusion strategy (MFS) for delivering CRISPR-Cas13a components into the exosomes [98]. Inside the exosome, CRISPR-Cas13a got activated by presence of target miRNA and resulted in generation of fluorescence signal on cleavage of reporter probes as shown in Fig. 5c. The signal could also be used to quantify the amount of target miRNA. The detection limit obtained was of 1.2×10^3 particles/mL with linear range of 10^4–10^8 particles/mL. It has lower sensitivity as compared to the reported work of Zhou et al. (2024) which although was able to detect *in situ* without disrupting exosomal structure. However, present study emphasizes signal confinement within fused vesicles, potentially offering more precise and accurate measurements. This study is also focused on miR-21, a well-established cancer biomarker, making it potentially more applicable in various clinical settings.

4.3. Miscellaneous CRISPR-Cas based biosensor for the detection of breast cancer

4.3.1. CRISPR-based lateral flow assay for the detection of breast cancer

Tian et al. (2024) introduced an innovative tandem CRISPR-Cas13a/Cas12a-based lateral flow assay (tanCRISPR-LFA) for the precise detection of miRNA-21, a potent biomarker for breast cancer. The biosensor used a locked RNA/DNA probe as a fuel [99]. On recognition of miRNA-21, the "unlocked" RNA got cleaved by Cas13a. This cleavage further resulted in activation of Cas12a, which then target and cleave a DNA sequence. These cleaved products of Cas13a and Cas12a were detected by a paper-based lateral flow strip. The presence of miRNA-21 was visible as signal development on the test line and control line. The type of sample investigated include cell extracts from MDA-MB-231 cells (breast cancer cell line). This highly advanced, portable, and efficient platform was able to detect minute levels of as low as 301 fM of miRNA −21. The paper-based lateral flow assay component of the system allows for visual detection of miRNA-21 and is suitable for point-of-care testing (POCT) in resource-limited settings. This novel and highly effective approach was able to address various limitations of current CRISPR-based assays, but still the integration of the LFA platform might face challenges regarding signal generation efficiency when dealing with complex biological matrices.

The use of quantum technology in form of quantum dots have been increasing in the field of cancer diagnostics. Lei et al. (2024) proposed a CRISPR-Cas12a-based lateral-flow assay incorporated with quantum dot encoded beads for the direct detection of *BRCA-1*. The biosensor used amplification free detection of the target *BRCA-1* by CRISPR-Cas12a [100]. The activated Cas12a enzyme then cleaved the neighboring ssDNA present on the quantum-dot encoded beads (QDBs). This cleavage was then detected using a lateral flow assay, where the cleaved

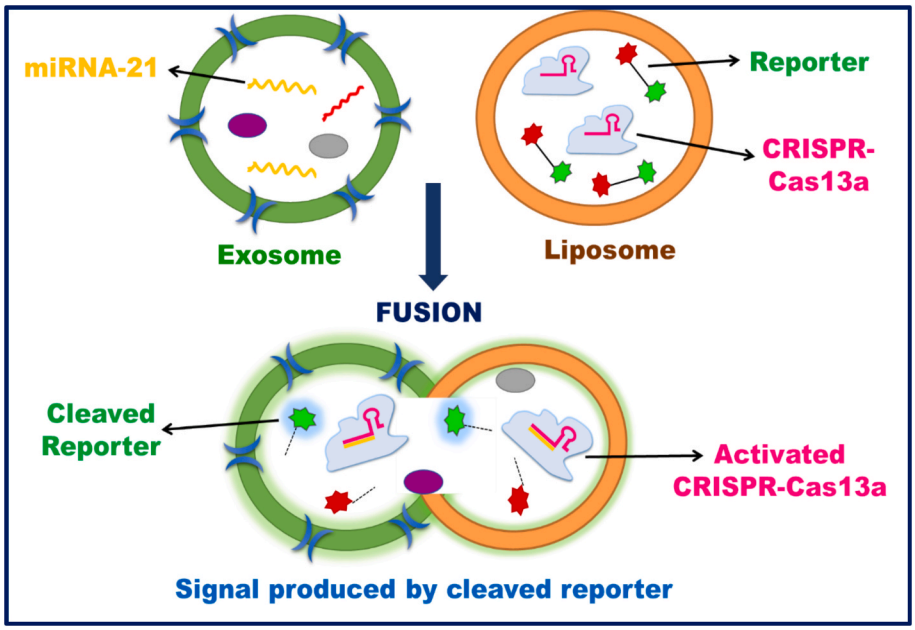

Fig. 5c. Schematic representation of liposome-based detection of breast cancer associated exosomal miRNA-21 by CRISPR-Cas13a based biosensor.

QDBs are captured and visualized on the test strip in the form of a detectable line as depicted in Fig. 5d. This biosensing platform was able to achieve detection limit of femtomolar level and was able to detect *BRCA-1* in less than 40 min. This biosensor was highly sensitive, amplification-free, rapid, visual, and user-friendly. It demonstrated the potential of use of quantum dots in the early and accurate diagnosis of breast cancer.

4.3.2. CRISPR and dark field microscopy-based biosensors for the detection of breast cancer

A CRISPR-Cas12a based biosensor combined with single nanoparticle dark-field microscopy (DFM) was developed by Li et al. (2024) for the detection of cfDNA associated with breast cancer. The biosensor detected cfDNA by monitoring the changes in the aggregation state of gold nanoparticles (AuNPs) [101]. Two sizes of AuNPs were used, 50 nm (AuNPs1) and 20 nm (AuNPs2). AuNPs1 were functionalized with a double-stranded DNA (dsDNA) probe and adsorbed onto a coverslip. In the presence of target cfDNA, *BRCA1* in this case, CRISPR-Cas12a was activated and unhybridized ssDNA portion of the dsDNA probe attached with AuNPs1 got cleaved. Without target DNA, AuNPs2 got bound to AuNPs1 via the dsDNA, causing aggregation, which resulted in the shift of the colour of scattering under DFM from green to yellow or red. Whereas, in the presence of target DNA, cleavage by Cas12a prevent this aggregation, resulting retention of green scattering points. The mean shift and partial least-square (PLS) algorithms were used to quantify the proportion of monomeric (green) versus aggregated (yellow/red) AuNPs, correlating with the concentration of the target BRCA-1 cfDNA. The sample investigated included cell lysates from breast cancer cells (MCF-7) and human embryonic kidney cells (HEK293). The working principle of this fabricated biosensor is represented using Fig. 5e. The

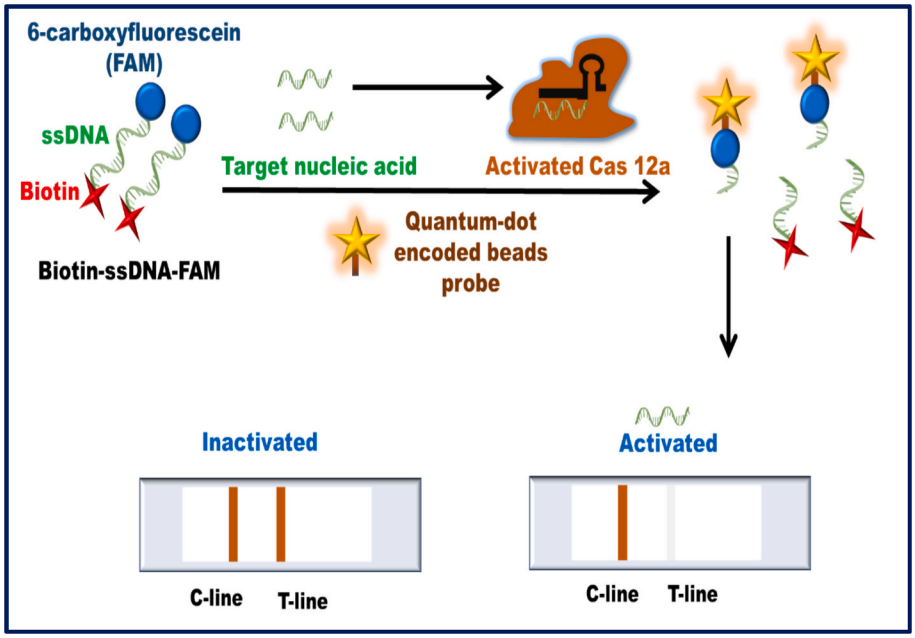

Fig. 5d. Diagram illustrating amplification free detection *BRCA-1* by using quantum-dot encoded beads by CRISPR-Cas12a based lateral flow assay.

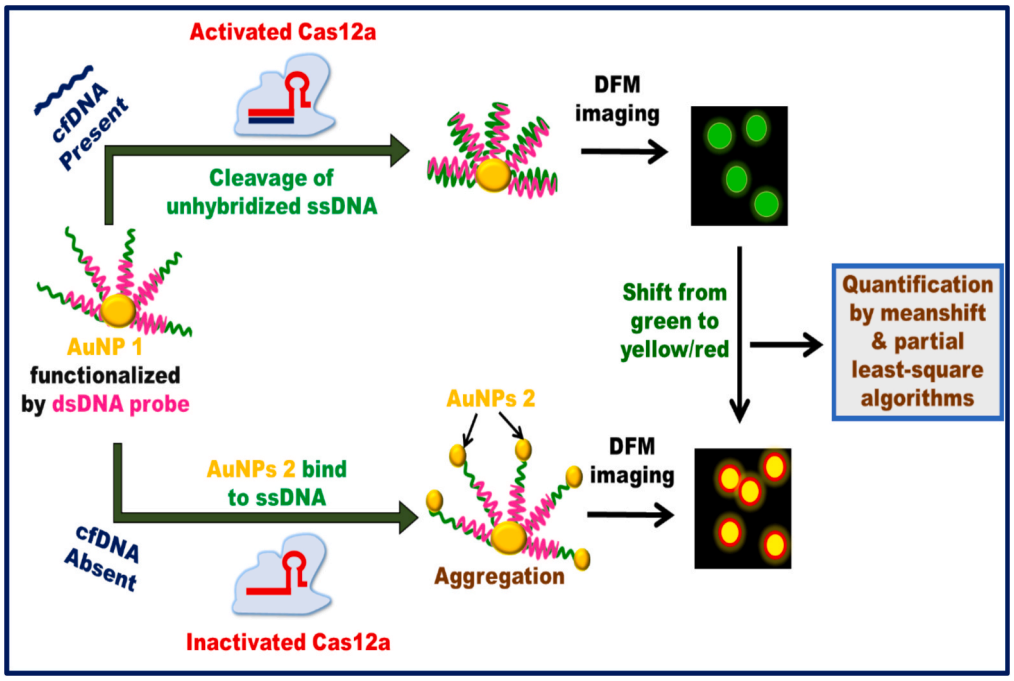

Fig. 5e. Schematic illustration of detection of target BRCA-1 cfDNA by dark field imaging based CRISPR-Cas12a biosensor.

biosensor was able to report a very low detection limit of 0.081fM and a linearity range of 0.1 fM to 500 pM for the *BRCA-1* gene. The detection was also rapid and was completed within 40 min. However, it would require sophisticated image processing techniques, which would increase the complexity and cost of the analysis. Also, there is a chance of potential interference when used with other complex biological matrices, such as blood. Despite, this CRISPR-Cas12a-based biosensor offers a novel and highly sensitive method for detecting cfDNA, with ultra-high sensitivity and the potential for real-time analysis in the clinical settings.

Similarly, another dark field imaging biosensor was developed by Kim et al. (2024) for the detection of miR-21-5p, a key biomarker in breast cancer. This biosensor integrated target recognition by CRIPSR-Cas13a with magnetic gold nanoparticles (MGNPs) and portable dark-field (DF) imaging for signal detection [102]. The detection mechanism involved binding of target miR-21-5p to crRNA hence, activating Cas13a enzyme. This activation resulted in the cleavage of RNA linker in MGNPs complex, resulting separation of gold nanoparticles (AuNPs) from magnetic beads. These released AuNPs in proportion with target miRNA, were then captured on a glass slide and quantified by portable DF imaging system. The biosensor was able to achieve a LOD of 500 aM and linearity range of 10 fM to 1 nM. The total detection time was determined to be of 30 min with, 10 min of CRISPR-Cas13a reaction and 18 min of AuNPs conjugation and imaging. The biosensor was found to be highly specific and could even distinguish single-base mismatches, validated against sequences such as miR-421 and miR-9-5p. The compact and cost-effective design of this biosensing platform is suitable for point-of-care settings hence offering a potential tool for breast cancer diagnostics.

4.3.3. CRISPR-based advanced and multimodal biosensor for the detection of breast cancer

With the advancements of technology, scientific community is focused in making diagnostic tools portable and more accessible. One such initiative was taken by Shi et al. (2024) by designing a smartphone-assisted CRISPR-Cas12a based biosensor for the detection of microRNA-let-7a, a breast cancer biomarker. The biosensor used 3D DNA walkers that moved along a DNA track and released trigger strands that further activated CRISPR-Cas12a system [103]. The activated Cas12a cleaved ssDNA linked to enzymes, resulted in reduced enzyme loading on the bioanode and decreased electron transfer. Capacitors were also integrated to amplify the detection signal and sensitivity of the biosensor. This platform can be operated through a dual-mode approach and utilized both electrochemical and colorimetric output. The decreased electron transfer led to reduced open-circuit voltage (EOCV) in the electrochemical mode, while a lighter colour in the colorimetric mode was detected by a smartphone as shown in Fig. 5f. The fabricated biosensor used biofuel cell electrodes that were modified with gold nanoparticles (AuNPs) on ultra-thin 2D graphdiyne (GDY) to enhance electrochemical signal detection. The miRNA-let-7a was detected with high sensitivity with LOD of 8.11 aM and linearity range of 0.0001 to 10,000 pM. This biosensor however was complex in design but the use of enzyme biofuel cells (EBFC) and capacitors provided a self-powered system, which reduced the need for external power sources and hence enhanced portability. The smartphone integration further allowed for on-site detection and remote disease sensing, facilitating real-time data analysis and transmission.

In another study by Cui et al. (2023), a multimodal CRISPR-Cas12a based biosensor was fabricated. The biosensor used a dumbbell DNA structure with a 5′ end flap designed to be specifically cleaved by FEN1 [104]. The cleavage created a site for ligation by T4 DNA ligase, forming a complete duplex DNA with a protospacer adjacent motif (PAM). This PAM was then recognized by CRISPR-Cas12a system and activated its *trans*-cleavage activity on ssDNA. In the fluorescence mode detection, the ssDNA was labelled with a fluorophore and a quencher and generated a fluorescence signal upon cleavage by Cas12a. For turbidimetric detection mode, the ssDNA connected magnetic microparticles (MMPs) and polystyrene microparticles (PMPs). Cleavage by Cas12a resulted increased levels of free PMPs, altering solution turbidity after magnetic separation. For the microfluidic detection, free PMPs were separated and collected in a microfluidic chip, here the visible length of PMP accumulation is in proportion with FEN1 activity. This biosensor was highly sensitive, specific, rapid with multimodal detection. The combination of fluorescence, turbidimetric, and microfluidic detection modes allows for versatile applications of the reported biosensor, particularly in breast cancer diagnostics.

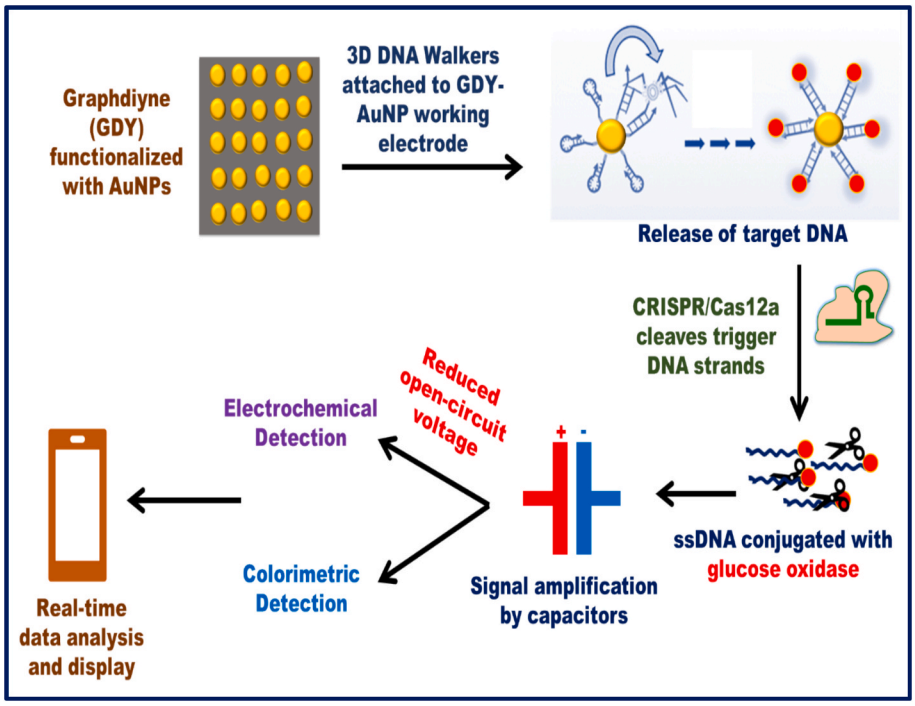

Fig. 5f. Diagrammatic representation of microRNA-let-7a detection by using a smartphone-assisted, CRISPR-Cas12a and 3D DNA walker mechanism based biosensing platform.

A detailed and comparative analysis of some of the other notable CRISPR-based biosensors for the detection of breast cancer is provided in Table 4.

By reviewing the aforementioned applications of newly reported CRISPR- based biosensing strategies for breast cancer diagnostics we can infer that, CRISPR-Cas based biosensing have developed to become a state-of-the-art and future-ready analytical approach. Recent advancements in CRISPR- integrated nano biosensors have enhanced the potential of their use in breast cancer detection. For instance, the development of multiplexed biosensors which enables simultaneous detection of miR-21, ATP, and hOGG1 has enabled versatile detection and comprehensive profiling [111]. Further integration of nanomaterials like gold nanoparticles, graphene oxide, and mesoporous nanozymes has resulted in improved signal detection and achieving low detection limits. For instance, in a study by Divya et al. (2023), the selection of electrode materials and the functionalization techniques have played an important role in enhancing sensitivity and performance of the biosensor. This biomimetic bilayer lipid membrane biosensor functionalized with 2D MXene nanosheets was able to detect BRCA1 gene in zepto molar ranges [119]. Portable CRISPR- based biosensors are also being developed which are emerging as a cost-effective, rapid, sensitive, and point-of-care diagnostic solutions for breast cancer. The incorporation of advanced techniques such as, dark field imaging is also providing a solution for real time analysis of breast cancer biomarkers with high sensitivity and accuracy. Furthermore, integration with smartphones and fabrication of microfluidic devices has also opened the possibility of the use in real-time scenarios as point-of-care devices.

5. Challenges associated with CRISPR-Cas based biosensing techniques for breast cancer detection and potential solutions

Despite the promising potential of CRISPR-based biosensors, early stage cancer detection faces significant hurdles like need for target amplification, low repeatability, and lack of awareness [75]. One of the key challenges is the identification of reliable biomarkers. As breast cancer is a heterogenous disease with multiple types and subtypes, each of which is expressing different molecular profiles. Hence, identification of a single biomarker specific for all types of breast cancer is a challenge. Moreover, these biomarkers are present in minute quantities, can share similarities with non-cancerous proteins, or can even fluctuate during the treatment process, leading to increased chances of false positives or false negatives. To address this challenge, focus should be made on enhancing the selectivity and specificity of gRNA sequences, improving signal amplification by using advanced nanomaterials, and employing multiplexed detection for more than one biomarker detection. This will not only increase specificity and sensitivity of biosensor but will also improve the detection accuracy.

Integration of CRISPR with nanomaterials in a biosensor can cause certain issues such as interference from nanomaterial properties, unintended steric hinderance, design complexities, or reduced stability of CRISPR-based systems over time [6]. Advances in nanotechnology, to develop biocompatible, stable, self-assembled, and novel nanomaterials is the need of the hour.

Other key challenge arises in terms of scalability of CRISPR-integrated biosensors from the lab to real-world applications. The development of complex CRISPR-based biosensors is costly and difficult to construct in large quantities. Further, CRISPR systems may require specialized technique to use and can cause a barrier to use in low resource settings. Also, the cumbersome process of sample preparation, data collection, need of specific reagents, and potential false positive detection hampers the application of CRISPR-biosensors. Fabricating cost-effective and miniaturized biosensors is the key to improve reproducibility and scalability.

Also, the ethical concerns of using CRISPR in diagnostics should be considered and genetic privacy in healthcare must be ensured. Clear, well-defined guidelines for the use of CRISPR-biosensors in human diagnostics must be framed before the widespread introduction.

6. Future prospects

The future of breast cancer diagnostics lies in the early and accurate diagnostics by converging advanced molecular detection strategies, biosensing techniques, and precision medicine. With the ongoing advancements in CRISPR-based biosensing there is an immense potential

Table 4
A comparative analysis of prominent CRISPR-based biosensors for the detection of breast cancer.

Type of biosensor	CRISPR-Cas System	Detection mechanism	Type of electrode used	Nanomaterial used	Detection target	LOD	Linearity range	Detection time	Special feature	References
Nucleic acid biosensor	CRISPR-Cas13a	The biosensor utilized the *trans*-cleavage activity of Cas13a, which is activated by the target miRNA, leading to the unfolding of hairpin structures and subsequent signal amplification via Primer Exchange Reaction (PER) concatemers.	Gold electrode modified with hairpin 2 (HP2) and methylene blue (MB) tagged DNA.	–	miR-21	30.2 fM	10^{-13} to 10^{-7} fM	~1 h	The biosensor targeted amplification-free detection and simplified the detection process.	[105]
Electrochemiluminescent (ECL) biosensor	CRISPR-Cas12a	The biosensor detected exomiRNA-155, by combined use of the amplification capability of 3D walking nanomotor and the CRISPR-Cas12a system. Mesoporous nanozyme-modified DNA tetrahedrons further provided enhanced electrochemiluminescent signals.	–	Mesoporous Nanozymes (TCPP-Fe@HMUiO@Au-ABEI)	exosomal microRNA-155	0.2732fM	1.0 fM to 1.0 nM	–	The combination of CRISPR/Cas12a with 3D walking nanomotors and ECL-active nanozymes represented an innovative approach to ultrasensitive miRNA detection	[106]
Nucleic acid biosensor	CRISPR-Cas13a	The biosensor detected miRNA-21 through a CRISPR-Cas13a-mediated catalytic hairpin assembly (CHA), which led to an amplified electrochemical signal that corresponds to the presence of miRNA-21.	Gold (Au) electrodes, functionalized with hairpin DNA probes	–	miRNA-21	2.6 fM	10 fM to 1 nM	–	Modular design of this biosensor allows for the multiplexed detection of other miRNAs by changing the spacer sequence in the Cas13a system.	[107]
Impedimetric biosensor	CRISPR-dCas9	The detection method for this biosensor involved binding of ctDNA with dCas9-sgRNA complex on a modified graphene oxide electrode. This binding was then measured as a change in impedance through Electrochemical Impedance Spectroscopy (EIS).	Graphene oxide screen printed electrodes (GPHOXE)	Graphene Oxide	ct-DNA from PIK3CA exon 9 mutation	6,50,000 fM	20,00,000–2,00,00,000 fM	40 s	This is the first study reported to use CRISPR/Cas technology for detection of PIK3CA exon 9 mutations in ctDNA's in a label-free impedimetric system.	[108]
Portable Electrochemiluminescence (PECL) Chip	CRISPR-Cas13a	In this biosensing platform, miRNA was detected by combining the Cas 13a's RNA recognition and cleavage activity with electrochemiluminescence.	Paper-Based Bipolar Electrodes (pBPE)	Light-Switch Molecule [Ru $(phen)_2dppz]^{2+}$	miRNA-17	1 fM	1 fM – 100 fM	–	This study is one of the first to integrate CRISPR/Cas13a with ECL technology in a portable chip format for miRNA detection.	[109]
Colorimetric and fluorescence dual biosensor	CRISPR-Cas12a	The biosensor detected the breast cancer biomarker BRCA1 by monitoring changes in both colorimetric and fluorescence signals, which had occured due	–	–	*BRCA1*	Colorimetric method: 6,15,000 fM; Fluorescence method: 2,89,000 fM.	–	–	This is a dual mode biosensor, based on CRISPR-Cas12a system for enhanced specific and sensitive	[110]

(*continued on next page*)

Table 4 (*continued*)

Type of biosensor	CRISPR-Cas System	Detection mechanism	Type of electrode used	Nanomaterial used	Detection target	LOD	Linearity range	Detection time	Special feature	References
		to the collateral-cleavage activity of CRISPR-Cas12a.							detection of *BRCA1*.	
Optical biosensor	CRISPR-Cas12a	The biosensor used a specially designed activator strand (AS) to reduce background noise. The AS can inhibit and subsequently reactivate the CRISPR-Cas12a enzyme hence, causing sensitive detection of various targets within live cells.	–	–	miRNA (miR-21); ATP (Adenosine Triphosphate); hOGG1 (Human 8-oxoguanine DNA glycosylase 1)	miR-21 = 960 fM; ATP = 8.6×10^9 fM; hOGG1 = 8.3×10^{-5} U/mL.	–	–	This biosensor is effective for real-time imaging and analysis of biomolecules in live cells.	[111]
Fluorescence biosensor	CRISPR-Cas12a	This biosensor detected miRNA directly through the *trans*-cleavage activity of CRISPR-Cas12a without the need to convert miRNA to cDNA. The detection was further enhanced by using gold-nanobeacon reporters.	–	Gold nanoparticle-based molecular beacons	miRNA-21	–	–	5 min	This study for the first time reported the activation of *trans*-cleavage activity of CRISPR-Cas12a directly by miRNA, without the need for conversion to cDNA.	[112]
Fluorescence biosensor	CRISPR-Cas12a	The detection mechanism of this biosensor involved the amplification of target signal with isothermal exponential amplification reaction (EXPAR) and further amplification and fluorescence signal generation by activation of CRISPR-Cas12a system.	–	–	miR-27a	0.09 fM	0.1 fM- 1,00,00,000 fM	60 min	This biosensor demonstrated high specificity, as it effectively distinguished miR-27a from other microRNA family members and base mismatches.	[113]
Optical biosensor	CRISPR-Cas12a	The biosensor used activation of CRISPR-Cas12a by target cell-free DNA (cfDNA), which led to the degradation of single-strand DNA (ssDNA) between gold nanoparticles (AuNP) and a fluorophore, resulting in metal-enhanced fluorescence (MEF) and a visual color change.	–	Gold nanoparticles (AuNP)	cfDNA with focus on breast cancer gene-1 (*BRCA-1*)	0.34 fM	1 fM to 1,00,000 fM	30 min	This dual-mode biosensor involved amplification-free detection, with reduced risk of false positives and simplified assay.	[114]
Fluorescence biosensor	CRISPR-Cas12a	The biosensor used a combination of cascaded strand displacement amplification (C-SDA) and the *trans*-cleavage activity of CRISPR/Cas12a.	–	–	Gene-$PIK3CA^{H1047R}$ mutation	50 fM	Mutated gene to wild type gene ration from 0.0001 % to 50 %	~140 min	This biosensor has enhanced anti-interference capability. Also, it was able to detect the less abundant $PIK3CA^{H1047R}$ mutation with high sensitivity.	[115]

(*continued on next page*)

Table 4 (*continued*)

Type of biosensor	CRISPR-Cas System	Detection mechanism	Type of electrode used	Nanomaterial used	Detection target	LOD	Linearity range	Detection time	Special feature	References
Fluorescence and lateral flow-based biosensor	CRISPR-Cas12a	The biosensor detected telomerase activity by using the enzyme to extend DNA fragments, which then resulted in the activation of the CRISPR-Cas12a system to cleave ssDNA reporters. These reporters can then produce a fluorescence or lateral flow-based highly amplified signal.	–	–	Telomerase activity in MCF-7 cells	Fluorescence assay = 57 cells/mL and lateral flow assay = 5.7×10^2 cells/mL	–	1 h	The biosensor was not only able to detect telomerase activity but also had the potential of identifying and differentiating different breast cancer cell subtypes.	[116]
Fluorescence biosensor	CRISPR-Cas12a	The biosensor detected miRNA-21 by amplifying the target miRNA through a rolling circle amplification (RCA) process. The enhanced signal then led to the CRISPR-Cas12a mediated cleavage, resulting in fluorescence signal generation.	–	–	miRNA-21	34.7 fM	10 fM to 10,00,000 fM	RCA Amplification: 4 h; CRISPR/Cas12a Cleavage: 20 min	This biosensor was easy-to-operate due to the wash free character and provided enhanced sensitivity as compared to traditional RCA-based methods.	[117]
Fluorescence Biosensor	CRISPR-Cas13a	The biosensing platform used programmable crRNA (CRISPR RNA) guided Cas13a to the target miRNA further, activating Cas13a's *trans*-cleavage activity to generate a detectable signal.	–	–	miRNA-17	0.0045 fM	0.001 to 100 fM	30 min	This is a one-step assay and can accurately detect miRNAs with minimal false positives.	[118]

for revolutionizing breast cancer diagnostics. The CRISPR-based biosensors are now being more robust by incorporating the use of nanomaterials, dark field imaging, smartphone-assistance, and quantum dots incorporation. Further exploration and integration of artificial intelligence (AI) and machine learning (ML) have the potential of enhancing the breast cancer diagnostics by enhancing the accuracy, speed, and reliability of complex data analysis. The development of novel nanomaterials can not only enhance the detection but can also contribute to theranostic potential of the CRISPR-based biosensors. These biosensors can further be miniaturized and developed in a form of implantable and wearable biosensors which would provide with continuous and on-site monitoring of breast cancer biomarkers. Additionally, the CRISPR-based biosensing strategies can also be facilitated in the development of personalized breast cancer diagnostic and treatment that can offer tailored solutions depending on unique biomarker and genetic profile of an individual. Overall, with the significant developments in the field of molecular biology and oncology there is a great possibility of development of more precise, personalized, rapid, and non-invasive detection technique in the near future which would transform the diagnostic and management of breast cancer.

7. Conclusion

Breast cancer remains an important global healthcare burden involving complex interplay of various genetic, lifestyle, and environmental factors. The breast cancer pathogenicity has many aspects driven by key genetic mutations in tumor suppressor genes as well as in oncogenes with multiple signaling pathways involved. Various crucial biomarkers have been discovered with the search of new ones still going on. The demand for continuous exploration of new and reliable diagnostic tools has been continuously rising to enhance early diagnosis and personalized treatment strategies. Traditional breast cancer detection methods like mammography, ultrasound, MRI etc. have been widely used but they often suffer from various limitations in form of low sensitivity, false positives, false negatives, exposure to radiations, invasive procedures, and cost-effectiveness. The emergence of biosensing techniques have addressed numerous limitations of the conventional detection strategies. The development of biosensors is necessary to make breast cancer diagnostics fast, simple, low-cost, precise, and specific. Integration of nanomaterials further enhance the performance and accuracy of biosensors hence improving signal amplification and detecting breast cancer biomarkers at ultra-low level. CRISPR-based biosensors have gathered significant detection in the field of breast cancer diagnostics. They can detect specific genetic mutations or any other associated biomarkers with high precision. Traditional biosensors are often limited to detect less variety of biomarkers but CRISPR-based biosensors can be functionalized in detecting more than one biomarker by making only small changes. These biosensors also don't need any additional amplification steps and are useful for rapid diagnostics in clinical and point-of-care settings. Traditional biosensors may suffer from cross-reactivity and often lack multiplexed detection and flexibility. However, CRISPR-based biosensing strategies addresses these shortcomings and deliver highly accurate results. Despite their promising potential, CRISPR based biosensing techniques face challenges related to cost, regulatory approvals, adaptability in clinical settings, complex design, maintenance, and stability. These biosensors are still in their nascent stage and require further developments for routine clinical use. Further research and advancements in the field of breast cancer diagnostics is required for overcoming existing barriers and ultimately marking improvement in the breast cancer outcomes.

CRediT authorship contribution statement

Arzoo Saini: Conceptualization, Writing – original draft, Methodology, Formal analysis, Validation. **Neeraj Dilbaghi:** Validation, Formal analysis, Writing – original draft, Writing – review & editing. **Neelam Yadav:** Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Neelam Yadav reports administrative support was provided by Central University of Haryana. No conflict to anyone after its publication. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical Issues.

Not required.

Research data for this article

The data for this review article would be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2025.112908.

Data availability

Data will be made available on request.

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