

Label-Free Electrochemical Biosensors for Triple-Negative Breast Cancer Biomarkers: Emerging Recognition Strategies, Nanomaterials, and Clinical Translation

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Abstract: Triple-negative breast cancer (TNBC) is when the patient does not have the three most targeted molecules (estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 or HER2) and the patient resorts to chemotherapy (cytotoxic), and poor prognosis due to the fact that the three most targeted molecules are the ones that are monitored. There has been growing interest in the measurement of alternative circulating markers e.g. epidermal growth factor receptor (EGFR), soluble programmed death-ligand 1 (sPD-L1), exosomal PD-L1, CD44, mucin-1, tumour-derived microRNAs (miR-10b, miR-21, miR-652), and circulating tumour cells (CTCs) that do not require repeated tissue biopsies. Label-free electrochemical biosensors that can detect a binding event as a change in charge-transfer resistance, capacitance or redox current instead of a secondary enzymatic or fluorescent reporter provide a means of fast, inexpensive, and perhaps point-of-care measurement of these markers. In this review, the recognition strategies that have been used to impart selectivity to these platforms (antibodies, aptamers, molecularly imprinted polymers, peptides and nucleic-acid probes) and the nanomaterial transducers (graphene and its derivatives, 2D MXenes, metal and bimetallic nanoparticles, metal/covalent-organic frameworks) that have been used to amplify the signal generated by these platforms are reviewed. The platforms used to detect HER2, EGFR, PD-L1, CD44 and TNBC-specific miRs that were representative in the last 5 years are compared and the special lack of TNBC-specific multi-marker, clinically validated devices is highlighted. The review ends with a discussion of issues that present practical problems to address, namely sample-matrix fouling, recognition-layer stability, reproducibility/regulatory validation between the lab-scale sensors and the deployable point-of-care biosensors and diagnostics, and prioritizes the multiplexed clinically translatable TNBC biosensing.

Keywords: Triple-Negative Breast Cancer, Label-Free Biosensors, Electrochemical Impedance Spectroscopy, Aptamers, Nanomaterials, MXene, Liquid Biopsy, Point-of-Care Diagnostics

1. Introduction

Breast cancer is still the most prevalent cancer in women around the globe and triple-negative breast cancer (TNBC) makes up about ten to fifteen percent of all breast cancer and is a major cause of breast cancer deaths. TNBC is characterized as having none of the receptors (ER, PR and HER2) that are over-expressed in other types of breast cancer, and therefore, the treatments that have been so successful in certain breast cancer patients have not proved successful in patients with TNBC. This is mostly treated with cytotoxic chemotherapy and the disease has an earlier onset, more visceral and brain metastases and a shorter overall survival than hormone-receptor-positive disease.



One of the major challenges is the late diagnosis of TNBC. Today, the method is the same: imaging, then tissue biopsy, then the invasive, slow and cumbersome procedure of immunophenotyping for the absence of receptors, which would be a requirement for frequent monitoring to diagnose early recurrence or to monitor the evolution over time of the molecular profile of a tumour during treatment. This has led to an increased interest in the development of liquid-biopsy biomarkers i.e. proteins, nucleic acids and cells shed in the blood, blood serum or urine, and that, in principle, can be repeatedly and non-invasively sampled. Other candidates identified as relevant to TNBC include exosomal cargo (CD44 and mucin-1, both of which are markers of stemness) and a panel of microRNAs (miR-10b, miR-21 and the TNBC associated miR-652) present in circulation and related to tumour behaviour.

For electrochemical biosensors, the molecular recognition event can be directly converted into an electrical signal and these markers are expected to be easily detectable due to the fact that they can be measured with relatively simple, smaller and easily miniaturized instrumentation. In this family, the label-free formats are of special interest that detect the binding event, either as a change in double-layer capacitance, or as a change in redox peak current measured by differential pulse or square-wave voltammetry (DPV/SWV) or change in charge-transfer resistance measured by electrochemical impedance spectroscopy (EIS), without requiring a secondary labelled antibody, enzyme, or redox reporter. It eliminates labelling step, reduces assay time, reduces reagent cost, makes the way towards multiplexed/point-of-care devices.

The purpose of this review is to discuss the label-free electrochemical biosensors of biomarkers which are relevant to TNBCs in the past five years or so. It systematically summarizes the biorecognition methods used for achieving selectivity, the nanomaterials used to increase the transduced signal, some of the most recent platforms and reported analytical performance, and the barriers for bringing this highly aggressive breast cancer subtype to a point of care device.

2. Tnbc Biology And The Circulating Biomarker Landscape

TNBC is not a straightforward disease. Most importantly, transcriptomic profiling studies like the Lehmann classification have subdivided TNBC into four different subtypes: basal-like, mesenchymal, immunomodulatory and luminal androgen-receptor, and these have some overlapping patterns of driver alterations and surface protein expression. This heterogeneity is one of the reasons a single biomarker will not be suitable for diagnosis or monitoring, and is a common argument in the literature for the use of multiple markers, or multi-marker sets, in biosensing as opposed to a single analyte assay.

The expression of EGFR has been studied in many basal-like TNBCs as a potential protein biomarker and for multiplexed detection with other cell surface markers, such as ICAM-1, either as a marker per se or as a component of multiplexed protein detection assays. In particular, PD-L1 in the membrane-bound and soluble/exosomal forms is of particular importance as a marker for the advent of checkpoint-inhibitor therapy for PD-L1 positive TNBC and an electrochemical assay to quantify concentrations of exosomal or circulating PD-L1 has a direct impact on diagnosis and on selection and monitoring therapeutic response. Exosomes and CTCs, which are abundant on the surface of the vesicle or cell in comparison to blood cells, are typically enriched with CD44 and mucin-1 and therefore these markers are often employed for capture or identification.

In the case of nucleic acids, several microRNAs are suggested as specific to TNBC. The miR-10b is said to be an early marker of the metastatic potential; miR-21 is an oncomiR that is widely dysregulated in various cancers including breast cancer; miR-652 has been said to be differentially expressed in TNBC and has been directly screened using a paper-based screen-printed electrode platform. Apart from protein and microRNA targets there are circulating tumour DNA (ctDNA) with TP53 or BRCA1/2 mutation and circulating tumour cells (CTCs) providing the backdrop to biomarkers, but with greater pre analytical challenges: the absolute level of ctDNA is small and CTCs require size and/or affinity enrichment.

Both of these two different analytes possess a property that can be measured electrochemically: binding to the surface of an electrode, which includes the binding to a complementary recognition element, will result in a change in

the properties of the interface (steric exclusion of a redox probe to the electrode surface will increase the charge-transfer resistance, whereas a double-stranded nucleic-acid will alter the local charge double-layer capacitance at the electrode interface). That is why some transduction techniques keep reoccurring in different sensors, such as those for HER2, EGFR, PD-L1, CD44 and microRNA, and that's in part why electrochemical transduction is, in principle, agnostic to the analyte class.

3. Recent Label-Free Electrochemical Platforms: Literature Review

In the last five years, a host of label-free electrochemical platforms targeting the breast-cancer biomarker have emerged, some of which are directly applicable to TNBC, even if they did not specifically validate the platform on a patient population with TNBC. Hansen et al. reported a graphene modified electrode using 1-pyrene butanoic acid succinimidyl ester (PBSE) crosslinking of the graphene to a single-stranded DNA probe complementary to miR-10b that was used for hybridization detection by EIS, the sensor achieved a limit of detection near 10 pM, discriminated the target from similarly sized non-target microRNAs, and was validated directly in human serum, one of relatively few TNBC labelled studies to include real-sample testing.

On the protein side, Hosseine, Naghib and Khodadadi describe a green-synthesized reduced graphene oxide/Fe₃O₄/Nafion/polyaniline nanocomposite, for detection of the SKBR3 breast-cancer cell line through the expression of its HER2 protein, with cyclic voltammetry, EIS and square-wave voltammetry characterising each modification step, which they reported could reach a linear range of detection from about 10² to 10⁶ cells per millilitre, and a detection limit of around 5 cells per millilitre, which shows how magnetic and conductive-polymer nanocomposites can be combined to build sensitivity into a purely label-free format. There is an almost identical approach for MXene modified electrodes for cytosensing with similarly low levels of cells per millilitre, thereby further solidifying the 2D nanomaterials as the platform of choice for label-free cell-based sensing of breast cancer.

In the exosomal-protein domain, a few groups have concentrated on soluble PD-L1 and exosomal PD-L1, as these have direct relevance to the use of checkpoint inhibitors. Near 0.143 pg/mL detection limits were obtained for soluble PD-L1 by functionalizing an aptasensor with a covalent-organic-framework/carbon-nanotube composite impregnated with gold nanoparticles, and the ability to discriminate between different breast-cancer subtypes at the level of exosomes was directly demonstrated by using magnetic beads functionalized with CD63-aptamers to capture and enrich breast-cancer exosomes derived from cells of human MCF7 breast-cancer cell line, followed by separation of the PD-L1 from the MCF7 exosomes with an electrochemical readout. Two platform types based on graphene oxide/Prussian-blue probes and spiky Au@Fe₃O₄/aptamer capture units have been developed that so far have pushed the detection limit of exosome particles down to sub-attomolar particle number, without requiring any pre-processing, which is essential for future point-of-care applications.

The last, graphene quantum dots, were also quite popular: Kumar et al. introduced a graphene-quantum-dot platform for CD44, and Mohammadpour-Haratbar et al. reviewed a large number of graphene-based electrochemical biosensors for breast-cancer biomarkers, where the overall findings were that graphene derivatives in all cases showed superior electron-transfer kinetics and probe-loading density compared to bare carbon or metal electrodes. There are two reasons why it is significant that Raucci et al. demonstrated a paper-based screen printed electrode specifically for miR-652, a microRNA that was reported to be differently expressed in TNBCs: 1) the microRNA is a marker for TNBCs, and 2) the electrode is truly disposable and could be used in a point of care scenario. Olorundare et al. reported another nanodiamond/gold-nanoparticle immunosensor for HER2, which is another nanomaterial used in this field of application, apart from graphene, carbon nanotubes and graphene-like nanomaterials.

This is lacking throughout the bulk of this work, and is the inspiration for this paper. Although the studies were analytically impressive, they were not developed or validated in the context of TNBC per se but rather in general or in the context of HER2-positive breast cancer; the number of biomarkers covered (either protein, exosomal or microRNA); number of clinical samples used for validation (if any) was relatively small (spiked serum samples or single patient samples) compared to the multi-marker nature of TNBC biology described above; the performance metrics reported (LOD, linear range, repeatability) were reported in inconsistent units and matrices across studies

making direct comparison between the different designs difficult and time consuming, thereby delaying the identification of the most translatable platform design.

4. Recognition Strategies For Label-Free Sensing

The selectivity of any label-free electrochemical biosensor is almost solely dependent upon the biorecognition layer that performs the specific binding event; the transduction step, which measures the impedance, capacitance or the redox current does not distinguish the specific binding event from the generic non-specific adsorption event unless the biorecognition element is highly selective. There are five approaches to recognition which are prevalent in the recent literature on breast-cancer and TNBC biomarker sensing (Table 1 compares them).

The most widely used protein targets are the antibodies, and at the present time, the most popular immunosensors rely on antibodies, because antibodies are clinically proven to be highly specific and high affinity towards the antigen against which they are directed. Their main disadvantages in the absence of a label in an electrochemical setting are the high cost, batch-to-batch variability, limited long-term thermal stability and the inability to control the orientation of the antibody on the electrode surface, because if the antibody is oriented at random, then the antigen binding site may be buried by the antibody and the sensitivity and reproducibility of the electrochemical response may be compromised.

Most of the aptasensors reviewed above utilize aptamer as the recognition element, making it a short, single-stranded DNA and/or RNA molecule that was in vitro selected using SELEX to bind to the target molecule with antibody-like affinity. They are not only chemically synthesized, and thus not requiring any biological production, but they are also very small – the number of aptamers that can be immobilized on the surface of a flat electrode is very high (except for the case of the cell), the binding event is very close to the electrode, which gives rise to a relatively large charge transfer resistance, capacitance change, per binding event, and they can readily be modified with thiol, amine or biotin anchors for oriented covalent immobilization. The main disadvantage of their design is the susceptibility to nucleolytic degradation in raw serum which has resulted in the use of chemically stabilized backbones, such as 2'-fluoro or locked, in newer designs.

A completely synthetic alternative is molecularly imprinted polymer (MIP), where the recognition cavities are created around the target molecule during the polymerization process. While attractive for its ease of cost and use at the point-of-care, imprinting large protein targets like HER2 or EGFR can be technically challenging and the efficiency of imprinting and the homogeneity of the imprint is more difficult to control than the affinity of aptamers and antibodies, which has so far hindered the use of MIPs for TNBC biosensing compared to smaller molecule targets.

The typical recognition approach for nucleic-acid biomarkers is the change in the signal upon Watson-Crick hybridization of complementary single-stranded DNA probes immobilized on nanomaterial modified electrodes, as in the case of miR-10b and miR-652 above. Peptide-based recognition elements are a relative newcomer which offer some of the stability benefits of small synthetic molecules, and which can have tunable affinity, and are starting to be considered as an alternative to full antibodies to surface-protein targets, where either the cost of an antibody or its stability is an issue.

Table -1: Comparison of biorecognition strategies used in label-free electrochemical biosensors for breast-cancer biomarkers

Recognition Element	Typical Target Class	Key Advantage
<i>Monoclonal antibody</i>	<i>Protein (HER2, EGFR)</i>	<i>Very high, well-characterized affinity</i>
<i>Aptamer (ssDNA/RNA)</i>	<i>Protein, exosome-surface marker</i>	<i>Low cost, small size, dense oriented packing</i>
<i>Molecularly imprinted polymer</i>	<i>Small protein, metabolite</i>	<i>Robust, reusable, inexpensive</i>

<i>ssDNA hybridization probe</i>	<i>microRNA / ctDNA</i>	<i>Simple, sequence-specific design</i>
<i>Peptide ligand</i>	<i>Cell-surface protein</i>	<i>Stable, tunable, low production cost</i>

5. Nanomaterial-Enabled Signal Amplification

Most high performance label-free electrochemical biosensors require the use of a modified electrode that contains a nanomaterial, which improves the change in the electrical properties of the interface into a measurable change that can be used to provide a reliable signal. The platforms reviewed here share the overall architecture shown in Figure 1; the biomaterial to be detected is placed on a working electrode immobilized with a recognition layer onto which a nanomaterial is attached and a change in the surface occurs when the target binds the biomaterial, which is detected as a change in charge-transfer resistance, redox peak current or capacitance.

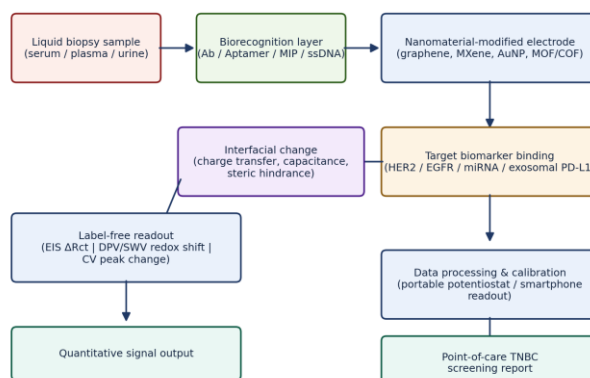


Fig -1: General architecture of a label-free electrochemical biosensor for TNBC biomarker detection

A schematic of a general architecture of a label-free electrochemical biosensor detecting biomarkers of TNBC is presented in Fig -1.

Graphene and its derivatives – graphene oxide, reduced graphene oxide and graphene quantum dots – are the most common nanomaterial family in this area, and are featured in some of the platforms mentioned above. Their very large specific surface area gives them high, uniform loading of probes and their high intrinsic electrical conductivity ensures good electron-transfer kinetics at the electrode surface which reduces the baseline charge-transfer resistance and thus increases the relative change in signal caused by a binding event. The above-mentioned miR-10b sensor uses π - π stacking to position probes, such as nucleic acids and proteins, in an oriented covalent manner on the surface of graphene for achieving improved reproducibilities in comparison to the conventional physisorption.

More recently, 2D materials known as MXenes, transition-metal carbides and nitrides like $\text{Ti}_3\text{C}_2\text{T}_x$, are emerging as an alternative and/or complementary 2D platform. They have hydrophilic terminated surfaces which promote functionalisation in the aqueous phase and a metallic level conductivity coupled with a very high surface-area-to-volume ratio, which has led to aptasensors with reported femtomolar detection limits of cancer protein biomarkers. The biggest hurdle they face in translating this technology is that the synthesis of large-scale, batch-reproducible graphene is still relatively early and the supply chain for graphene is more established.

Metal and bimetallic nanoparticles (most frequently Au nanoparticles, but other bimetallic structures can be prepared, e.g. PdCuB microporous nanotags or $\text{Au}@\text{CuCl}_2$ nanowires) as direct conductivity enhancers, or as tags to amplify the additional redox activity in a sandwich-type design at the location of the captured target. In particular, the presence of well-established procedures for orientational convenient thiol based bioconjugation of antibodies or aptamers makes gold nanoparticles interesting.

Recent PD-L1 and HER2 aptasensors use a newer class of highly porous, tunable nanomaterials: metal-organic frameworks (MOFs) and covalent-organic frameworks (COFs), which are characterized by having very high surface

area, tunable pore chemistry for probe loading, and, in several designs, intrinsic peroxidase-like catalytic activity that further amplifies the electrochemical signal without an added enzyme label. Signals are further amplified by magnetic nanoparticles (also aptamers coupled to nanoparticles) which are usually functionalized with capture antibodies or aptamers against exosomal surface markers such as CD63, but which are not used directly as a signal amplifier but instead are used as a sample preparation tool: the application of an external magnetic field allows tumour-derived exosomes or cells to be rapidly enriched and separated from the bulk of a serum/ plasma sample prior to the label-free electrochemical measurement, reducing matrix interference and shortening the overall assay time – a practically important feature for eventual POCD use.

6. Representative Platforms For Tnbc-Relevant Biomarkers

To representatively summarize label-free electrochemical platforms for TNBC relevant protein, exosomal and microRNA biomarkers reported in the last 5 years, as well as the nanomaterial transducer, detection method and analytical performance, a representative collection of platforms that satisfies these criteria were gathered and summarized as the representative label-free electrochemical platforms presented in Table 2. The detection limits as reported in different studies are in different units (appropriate for the specific target and sample for each platform) so the detection limit in each row of Figure 2 should not be compared numerically, but should be compared within each row to reflect the relative trends in detection limits within each platform in the reportable context.

Table -2: Representative label-free electrochemical biosensors for TNBC-relevant biomarkers (last five years)

Biomarker	Nanomaterial Platform	Technique
HER2 (SKBR3 cells)	rGO/Fe ₃ O ₄ /Nafion/PANI	CV / EIS / SWV
miR-10b	Graphene / PBSE-ssDNA	EIS
Soluble PD-L1	COF/CNT/AuNP + HRP cycle	Amperometric
Protein biomarker (general)	MXene aptasensor	EIS / DPV
miR-652 (TNBC)	Paper-based screen-printed electrode	DPV
CD44	Electrochemically exfoliated GQDs	DPV / EIS

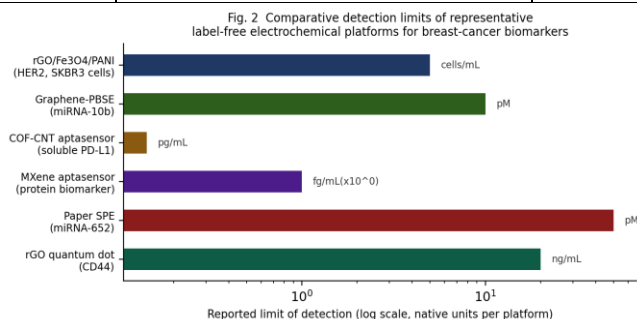


Fig -2: Comparative detection limits of representative platforms (native reporting units; relative visualization only)

There are two trends here to be noted. Furthermore, platforms available that are based on aptamers are the lowest in absolute detection limit reported to date for soluble and exosomal PD-L1 and the highest in the level of recognition element densities as well as the highest signal to matrix background level has been achieved using magnetic separation. Second, platforms based on cells and exosomes, which need to detect the presence of a much more abundant and much less abundant analyte, respectively, in a complex biological matrix, report detection levels

of several orders of magnitude higher in number of particles or cells, but are relevant in the clinic, because the levels of circulating tumour cells and exosomes in patient blood are generally low.

Altogether, these platforms demonstrate the availability of the individual components required to build a TNBC-tailored biosensing panel in the literature, including the detection of EGFR and PD-L1, detection of the TNBC-associated microRNAs and discrimination of the TNBC subtypes based on exosome analysis, but are developed and validated separately on different electrode platforms and not combined into a clinically validated multiplexed biosensing platform.

7. Clinical Translation And Point-Of-Care Considerations

Beyond the raw analytical sensitivity, there are multiple layers that need to be surmounted to make any of the platforms above work in the clinical or point-of-care setting. One such layer is the electrode format: screen-printed electrodes are less expensive, less reusable and can be used with small volume samples while the glassy-carbon electrodes require more complex handling and cleaning procedures for every sample. The easy attachment of such disposable electrodes to small battery-powered or smartphone-interface potentiostats is increasingly becoming the trend to shift the impedance or voltammetric readout from a specialized lab to a clinic and even a patient's home.

The 2nd layer is multiplexing. TNBC biology is also complex and is unlikely to be defined by a single biomarker that would make a good screening or monitoring device so multiple markers, including EGFR, soluble or exosomal PD-L1, and a variety of micro RNAs associated with TNBC would be an ideal choice to be measured simultaneously from a small volume of sample. The most common engineering approach to this problem reported in the wider literature is the incorporation of microfluidic channels through which the sample could be sequentially or simultaneously delivered to distinct spatially separated zones, each of which is differently functionalized, termed microfluidic integration; however, relatively few of the platforms that have been examined in the context of TNBC have been reported in a multiplexed format.

Sample-matrix complexity is the third barrier, and one that can be overlooked. The background level of protein, salts and other biomolecules in whole serum or plasma is very high and may cause a change in the detectable signal (impedance or capacitance) from the modified electrode due to non-specific binding of the biomolecules to the nanomaterial, unless careful control experiments are carried out, in which case specific binding of the target molecule may be hard to distinguish from a non-specific change in the signal (impedance or capacitance) from the modified electrode. Some of the mitigation strategies employed in the literature have been: using bovine serum albumin (BSA) or other blocking agents to block the steps; magnetic pre-concentration of the target away from the bulk matrix; the use of recognition layers that are highly specific to the target molecule and are known to have selectivity against structurally similar non-target molecules, as in the case of the miR-10b sensor which discriminated against similarly sized non-target microRNAs.

Lastly, there is a lack of analytical and clinical validation in the literature to support clinical translation: testing a statistically significant number of patient samples at various disease stages, comparison to a clinical reference method, demonstration of inter-electrode and inter-batch reproducibility, and validation of the shelf-life stability of the functionalized recognition layer under realistic clinical storage conditions. If the assay were validated to this standard, it will be applicable for a true application in the clinic beyond diagnosis, particularly for patients who are being evaluated for checkpoint-inhibitor therapy, as PD-L1 status – which the electrochemical assay could allow for rapid and cost-effective monitoring – will have therapeutic implications for these patients.

8. Limitations

As per the platforms reviewed below, there are certain limitations that temper the near-term clinical promise of these platforms. Essentially, none of the reported sensors is designed and/or tested specifically for patients with TNBC and most have been developed for breast cancer in general or HER2+ breast cancer, and their efficacy in receptor-negative and molecularly more diverse breast cancer has not been proven. They are typically reported in clinical

samples with external spikes or single source samples and are thus not representative of prospective and multi-centre patient samples; the true sensitivity and specificity and predictive value would not be known.

The same applies to the reproducibility of different batches of the same electrode, as well as between laboratories, especially when the electrodes are modified with nanomaterials which are sensitive to the small variations in the synthesis process, functionalization density and storage conditions, and the disparate units and reporting conventions across studies make direct comparison difficult and slow the development of the best design to focus on. Another challenge is the stability of the biorecognition layer, as both antibodies and unmodified aptamers suffer degradation under realistic storage conditions (thermally and by nuclease attack, respectively) and very few reports of data for the realistic storage conditions. Finally, many of the more sensitive nanomaterials discussed in this review, particularly MXenes and MOF/COF composites, lack well-established, batch-reproducible, large-scale synthesis procedures that are crucial for the cost-effective production of materials on a scale required for clinical applications on a wider scale.

9. Conclusions And Future Directions

In the past five years, the truly outstanding analytical sensitivity of label-free, judiciously designed electrochemical biosensors for detection of specific biomarkers relevant for TNBC that include proteins from femtomolar to single-digit cell/exosome counts has been demonstrated. The difficulty isn't the analytical sensitivity, which is pretty high, but the integration; the field hasn't yet gotten to a multiplexed panel of the assays that can be combined together to identify TNBC; to be validated on a realistic cohort of patients; to be built on a disposable format that is compatible with point of care; and to be characterised for the reproducibility and stability needed for use in the clinic.

Bringing the most sensitive recognition and nanomaterial strategies mentioned above, such as the aptamer/MOF channel for PD-L1 and graphene channel for microRNA, into a single screen-printed, microfluidic device, prospective validation on TNBC patient serum, and design implementation from the outset to consider shelf-life and manufacturing reproducibility are the next steps towards a practical and affordable point-of-care tool for TNBC screening and treatment monitoring. To close this integration gap, the clinical relevance is clearly more than just for diagnosis, as at least one of the biomarkers discussed here, namely PD-L1, has direct relevance for the treatment of TNBC patients

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