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Sensitive detection of unopposed estrogen using estrogen receptor functionalized nanoprobes for cancer diagnosis

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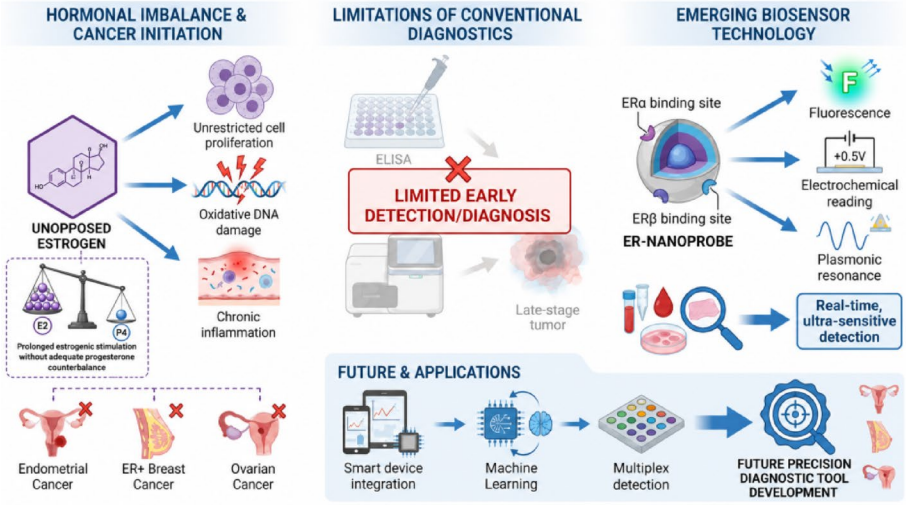
Abstract

Unopposed estrogen refers to prolonged estrogenic stimulation in the absence of adequate progesterone-mediated counter-regulation. Unopposed estrogen is strongly implicated in endometrial hyperplasia and type I endometrial carcinogenesis, and it is also biologically relevant to estrogen receptor-positive breast cancer. Evidence linking estrogen exposure to ovarian cancer is more heterogeneous and appears to vary by menopausal status, hormone therapy formulation, duration of exposure, and histologic subtype; therefore, ovarian cancer risk should be interpreted as an association rather than a definitive causal consequence of unopposed estrogen. Ongoing estrogen signaling can drive cell proliferation, oxidative DNA damage, and chronic inflammation in estrogen target tissues. Until now, conventional hormone assays, such as enzyme-linked immunosorbent assay (ELISA) have only been able to measure the amount of hormones in the circulation, but may not effectively detect dynamic, tissue-specific, or early-stage estrogenic activity. This review is unique in focusing on unopposed estrogen as a clinically significant biosensing target and critically reviewing estrogen receptor functionalized nanoprobe platforms for the detection of unopposed estrogen and the diagnosis of cancer, unlike other reviews, which have broadly discussed estrogen biosensors or nanoprobe-formatted cancer diagnostics. It focuses on platforms using estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) as well as strategies for receptor immobilization, nanomaterials design, signal transduction, and applications in biofluids, cells, and tissues. The review also discusses a translational aspect by considering obstacles to clinical implementation, such as probe stability, specificity of chemical analysis in complex biological matrices, required assay standardization, validation, and compatibility with use in a portable or point-of-care system. Newer trends like multiplex hormone profiling, smart devices integration, and signal interpretation with the help of machine learning (ML) are also discussed. This review aims to place unopposed estrogen biology in the context of ER-functionalized nanobiosensing and clinical translation problems, establishing a focused framework for future precision diagnostic tool development for cancer risk assessment and early detection by estrogen.

Keywords Unopposed estrogen, Estrogen receptor, Nanoprobes, Cancer biosensing, Early detection

Graphical abstract



1 Introduction

Unopposed estrogen is a physiological state in which estrogen levels are elevated or sustained without adequate progesterone [1, 2]. Progesterone normally counterbalances the proliferative effects of estrogen in estrogen-responsive tissues, such as the endometrium and breast epithelium [2]. In the event of this balance being upset, as in clinical situations such as anovulatory cycles, perimenopause, polycyclic ovary syndrome (PCOS) or with estrogen only hormone replacement therapy (HRT), estrogen effects continue without any check [2]. This chronic stimulation leads to hyperproliferation that can lead to genomic instability, chronic inflammation and progress to malignant transformation.

Unopposed estrogen has been strongly associated with carcinogenesis in several hormone-sensitive cancers [3]. Harmless estrogen (unopposed) has been most consistently associated with endometrial hyperplasia and type I endometrioid endometrial carcinoma, especially in conditions like chronic anovulation, obesity and estrogen-only menopausal hormone therapy [4]. Likewise, too much estrogen in breast tissue can cause the mammary epithelial cells to start multiplying and multiplying cells can be more susceptible to changes and development into estrogen receptor-positive breast cancer tissue [5]. An increasing body of evidence also reflects a possible relationship between long-term estrogen exposure and the development of ovarian cancer particularly in post-menopausal women [6, 7]. In contrast, link between the estrogen level and ovarian cancer is less certain. Evidence from large, epidemiologic, and meta-analytic studies indicate a moderate elevation in risk for Ovarian Cancer may exist with women taking Menopausal Hormone Therapy (MHT), but studies are heterogeneous and risk depends on the types of tumors, length of exposure, and the type of hormone therapy [8, 9]. Hence, a discussion of ovarian cancer as a possible estrogen related cancer, and not as a direct or proven outcome of unopposed estrogen is appropriate [10–12]. These observations highlight the need for improved monitoring of estrogen dominance to support early detection and hormone-based intervention.

Although of clinical significance, unopposed estrogen is a difficult condition to diagnose and follow precisely. The traditional methods of hormone measurement, such as immunoassays and ELISAs, provide only static measurements of serum estrogen and progesterone levels and do not capture dynamic hormonal activity or real-time fluctuations [13, 14]. Furthermore, such methods do not accurately reflect local activity of hormones or slight changes in hormones at an early stage [14]. Available screening tools for traditional cancer (mammography, pelvic ultrasound, endometrial biopsy) are usually applied when a person is already diseased or when one is already at high-risk, and therefore are not effective in preventing cancer [15–17]. Consequently, a number of instances of hormone-dependent cancer are detected at later stages of development when they are harder to cure.

Nanotechnology offers opportunities to overcome these limitations in hormone and cancer diagnostics [18–20]. Nanomaterials have distinct physicochemical properties with very large ratios of surface area to volumes, tunable nanoparticle-nanoparticle surface functionalities, and extremely high sensitivities to molecular interactions [21, 22]. These features qualify them as the perfect choice in biosensing applications. Nanoparticle-based sensors have been sensitive and specific in identifying proteins, nucleic acids as well as single cells in cancer diagnostics [22]. They can probe in real-time biomarkers of diseases through direct interaction with biomolecules, which in principle could advance the paradigms in early detection and enable more individualised healthcare [23–25]. Estrogen receptor-functionalised nanoprobes are one of the emerging nanobiosensing techniques that is considered a promising technique for detections of bioavailable estrogens, which are linked to unopposed estrogen exposure [26, 27]. The molecular recognition elements used with these platforms are estrogen receptor alpha (ERα) and/or estrogen receptor beta (ERβ), which are immobilized on the nanoprobe surface, but are not specifically binding fragments of each other. Instead, their ligand-binding domains are believed to detect and bind estrogen analytes, primarily 17β-estradiol (E2) which is the most potent and bio-active endogenous estrogenic compound [28–30]. These systems can be tailored for clinical or biofluid target to detect estrone (E1) which is particularly significant for postmenopausal estrogen exposure, or estriol (E3) which is more relevant to urinary estrogen profiling (extremely valuable for pregnancies) [31]. The interaction between estrogen and the ER functionalized nanoprobe can be translated into measurable optical, electrochemical, plasmonic, or impedance-based signals either by receptor–ligand interaction or ligand induced conformational changes. Therefore, the ER-functionalized nanoprobes provide a potentially sensitive and selective technique for monitoring the presence of the free or bioavailable forms of estrogen (especially E2) in biological fluid and could help facilitate early detection of estrogen-driven cancer risk [32, 33].

Although recent progress has been made in developing estrogen biosensors, the technology currently available is not comprehensive enough to meet the diagnostics needs for unopposed estrogen related cancer risk assessment. Many current biosensors or conventional hormone assays offer a static or an end-point reading and are unable to detect low levels or early-stage oestrogenic activity, or may cross-react with structurally related steroids and endocrine disrupting compounds or estrogen metabolites within complex biological matrices. Furthermore, the majority of platforms are still under development and not properly validated for real-time monitoring, non-invasive biofluid testing,

point-of-care applications and clinical translation. These restrictions constitute a large unexploited requirement for biosensors able to selectively detect bioavailable estrogens, to be able to measure the estrogenic ligand in the presence of other steroid hormones, to work in serum and urine or other body fluids (saliva, cells or tissues), and to provide a clinically interpretable signal for early cancer risk stratification. The unique advantage of ER-functionalized nanoprobes is a combination of the molecular recognition properties of ERα/ERβ and the high surface area, signal amplification, miniaturization potential and tunable optical, or electrochemical readouts of nanomaterials. In this respect, the goal of this review is to critically examine ER-functionalized nanoprobes for unopposed estrogen detection, focusing on ER immobilization, nanoparticles design, signal transduction, application in biofluids and tissues, the analytical selectivity, the prospects for real-time detection and the challenges that remain prior to clinical translation. First, the biological aspects of unopposed estrogen-mediated carcinoma are summarized, followed by a discussion on estrogen receptors as biosensing targets, nanoprobe design and functionalization options, detection modalities, clinical applications, challenges for translation and future directions.

1.1 Evolution and maturity of estrogen biosensing and ER-functionalized nanoprobe platforms

Estrogen biosensing has progressed in a number of sequential, but overlapping steps, from the traditional hormone quantification towards ever smaller, more selective and function-reading sensors. Previous evaluation took simplified methods such as immunoassays, enzyme-linked immunosorbent assays and subsequently, chromatographic or mass-spectrometric reference methods which allowed the quantification of circulating estrogens but provided little information about the real-time bioavailability, receptor interaction or local actions and/or early molecular changes linked to estrogen driven carcinogenesis [34, 35]. In addition, antibody, aptamer, and molecularly imprinted polymer based recognition systems were used in subsequent biosensor development for a faster biosensor, lower sample consumption, and transportability, while retaining challenges for cross reactivity, matrix effects, and separation of total estrogen levels versus active estrogenic signalling.

The second great transition was the coming of nanomaterials. Signal amplification, detection in small volumes, multiplexing capability and compatibility with optical, electrochemical, Surface Plasmon Resonance (SPR), Surface-Enhanced Raman Scattering (SERS), and microfluidic readout was possible by using gold nanoparticles, quantum dots, carbon nanotubes, graphene derivatives, magnetic nanoparticles, and plasmonic substrates [36, 37]. These advances made the detection of estrogen much more than a laboratory only measurement and introduced the possibility for potentially real-time and point-of-care detection. Estrogen receptor-functionalized nanoprobes have recently appeared as an extension of this trend as the estrogen receptors (ERs) ERα and ERβ offer a ligand-recognition interface that more closely mimics estrogen bioactivity than chemical detection alone.

Yet the field is far from homogenous in maturity, and there are still areas that are not mature. However, there are many ER functionalized platforms that are still in the process of proof of concept at the analytical or preclinical level with analysis of incomplete Limit of Detection (LOD), linear range, reproducibly, receptor stability, cross reactivity

and performance in clinically relevant matrices. The latest translation is towards microfluidic, wearable, multiplex and AI-assisted systems, which should be standardized, compared to reference methods, prospectively validated and evaluated by the regulators for routine diagnostics in the future [38, 39]. Hence, the evolution of the field is understood as a walk from quantification of hormones to enhancing the nanoscale signal to functional biosensing by receptor to clinical application which needs to be rigorously validated.

2 Unopposed estrogen and cancer development

2.1 Pathophysiology of the unopposed exposure to estrogen

2.1.1 Metabolism and body-wide effects of estrogen

Estrogen is synthesized primarily in the ovary, but also peripheral adipose tissues contribute to the synthesis of estrogens, especially in the post-menopausal females (Fig. 1) [5, 40]. The three main estrogens produced naturally in the body are here: Estrone (E1), Estradiol (E2), and Estriol (E3) with Estradiol being the most potent and biologically active. The bioavailability of estrogen is controlled by its synthesis, conversion in peripheral tissues by aromatase, hepatic hydroxylation and conjugation, and removal from plasma [41]. Thus, any factor that increases the activity of aromatase, that hampers the hepatic metabolism or that changes the phase I/II detoxification could lead to increased levels of circulating estrogens and to their accumulation of biologically active or genotoxic estrogen metabolites [5, 42]. These metabolic changes establish the biochemical basis for sustained estrogen exposure; however, carcinogenic risk increases further when progesterone-mediated counter-regulation is insufficient. Moreover, the estrogen receptors (ERα/ERβ), through which the majority of effects of estrogens are produced are variedly expressed in different tissues imparting the tissues to be highly responsive and easily affected by unopposed estrogen contact [43, 44]. As shown in Fig. 2, unopposed estrogen signaling contributes to hormone-sensitive cancer development through ER-mediated transcriptional activation, enhanced cell proliferation, survival signaling, inflammation, angiogenesis, and growth-factor pathway crosstalk.

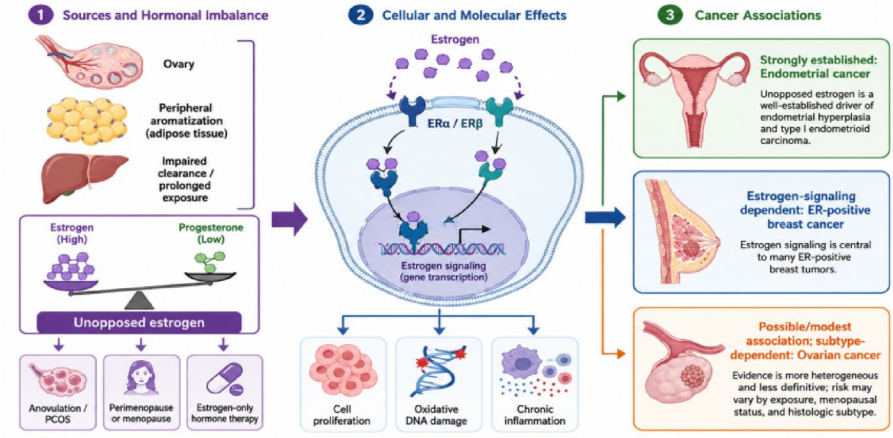


Fig. 1 Pathophysiological cascade of unopposed estrogen and evidence-graded associations with hormone-related cancers (Created in https://BioRender.com). This figure illustrates the systemic and cellular consequences of prolonged estrogen exposure without adequate progesterone counterbalance. Evidence strength differs across tumor types: the link between unopposed estrogen and endometrial hyperplasia/type I endometrioid carcinoma is well established, whereas ER-positive breast cancer reflects estrogen-signaling dependence, and ovarian cancer associations remain more heterogeneous, modest, and subtype-dependent

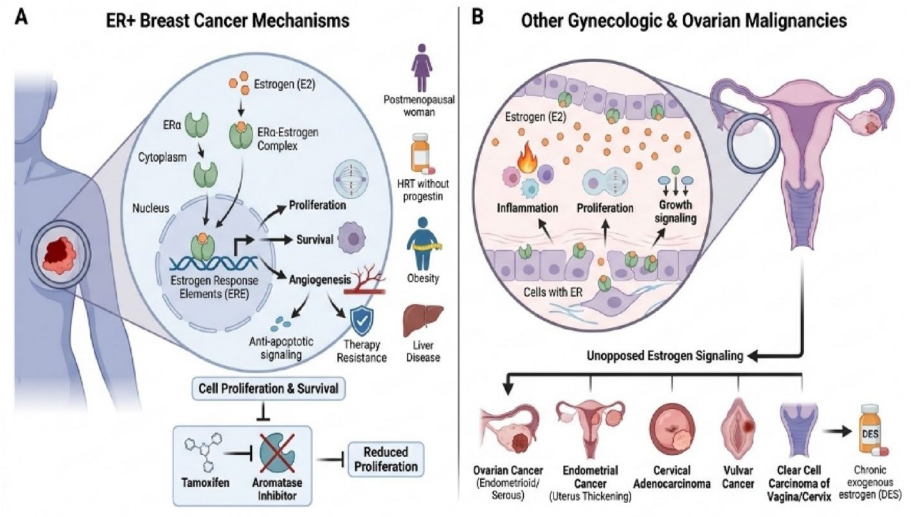


Fig. 2 Estrogen-driven mechanisms in ER-positive breast cancer and other gynecologic malignancies: Schematic illustration of how estrogen works in ER-Positive Breast Cancers: estrogen binds to ER α, activates ER α-mediated estrogen signalling and results in estrogen-response gene transcription, proliferation, survival, angiogenesis and anti-apoptotic pathways. This figure also outlines estrogen-related mechanisms in other gynecologic or ovarian malignancies which may involve inflammation, epithelial growth and proliferation and growth signaling in estrogen receptor-containing tissues due to unopposed estrogen stimulation. Risk clinical and biological situations are also mentioned: postmenopausal and obesity, liver disease, hormone replacement therapy without progesteron

2.1.2 Loss of progesterone-mediated regulation

Progesterone in the luteal phase of normal menstrual cycle opposes the effects of estrogen which induces proliferation of the endometrial tissue, by keeping the tissue differentiated and preventing excessive growth [45, 46]. Nevertheless, during anovulatory cycles, menopause, polycystic ovary syndrome (PCOS), or hormone replacement therapy (HRT) that does not contain progestins, estrogen is unopposed [46]. In the absence of the regulatory effect of progesterone, the mitogenic effect of estrogen will go unregulated and a proliferation of the endometrial and other estrogen-sensitive epithelial tissues will occur [47, 48]. The resultant hormonal imbalance interferes with normal cellular balance and increases the potential susceptibility of developing hyperplasia, atypia, and malignant transformation. Additionally, progesterone normally suppresses estrogen receptor expression and promotes estrogen-metabolizing enzymes; when progesterone is deficient, persistent receptor activation and prolonged estrogenic stimulation may occur [48]. This fact underlines the known connection between unopposed estrogen and carcinogenesis of the endometrium.

2.1.3 Estrogen-induced mitogenesis and DNA damage

Estrogen receptor activation by continuous estrogen exposure is the predominant mechanism by which mitogenic signaling is promoted, resulting in an increase in transcription of cell-cycle genes like cyclin D1 and c-Myc [49, 50]. With cell division occurring unabatedly replication errors and mutation build-up is enhanced especially in oncogenes and tumor suppressor genes. The redox cycling of estrogen metabolites particularly catechol estrogens, facilitates the production of reactive oxygen species (ROS) which destroy DNA directly, and also block DNA repair [51]. Estrogen receptors (ERs), especially ERα and ERβ, are formed to mediate a variety of estrogentic effects and become differently expressed in each tissue, resulting in the differential responsiveness to estrogen

exposure. Estrogen bound to the receptors triggers genomic and non-genomic pathways that control proliferation, differentiation, apoptosis and inflammation. Estrogen action continuously leads to the activation of transcription of cell-cycle genes including the mitogenic genes cyclin D1 and c-Myc in estrogen target tissues. Further enhancing the proliferative and anti-apoptotic responses can also enhance the crosstalk between estrogen receptor signaling and growth factor pathways, such as IGF-1 and HER2 signaling [52, 53]. This synergy enhances clonal proliferation of transformed cells which contributes to development and advancement of tumor cells, especially in estrogen sensitive tissues.

2.2 Estrogen-related cancers

2.2.1 Endometrial cancer

Endometrial cancer is the most well-characterized malignancy associated with unopposed estrogen exposure [54]. Epidemiological studies have always reported elevated risks among women with chronic anovulation, obesity or unopposed estrogen replacement therapy [55]. The presence of estrogen promotes the endometrial surface and without the progesterone, causes continuous growth with a possibility of hyperplasia [54]. This hormonal-imbalance is especially linked to type I endometrial cancer that is estrogen dependent and is usually low grade [56]. The patterns of genetic alterations that are typical of this cancer subtype are genomic instability, microsatellite mutation and PTEN mutation, which can be stimulated by the effects of the mitogenetic and mutagenic properties of high estrogen [57]. Moreover, the presence of estrogen receptors in the tissues of the endometrium allows the cells to be highly responsive to the hormonal stimulation, which increases the risk [58, 59]. Clinically, these tumors tend to initiate early momentous discharge of the uterus and it is one of the commonest manifestations and therefore, hormonal balancing is one of the primary methods to prevent them.

2.2.2 Estrogen receptor-positive (ER +) breast cancer

Another type of malignancy that is closely associated with the case of estrogen dominance is the Estrogen receptor positive breast cancer [5, 20]. Estrogen receptor alpha (Era) is over-expressed in these tumors and when these cells bind to estrogen there is induced expression of the gene pattern which foster proliferation, survival and angiogenesis [60]. The women who are postmenopausal, particularly those taking hormone replacement therapy (HRT) without progestin, and those with elevated endogenous estrogen levels secondary to obesity or liver disease are at high risk [61, 62]. Estrogen mediates the proliferation of ER + breast epithelial and creates the microenvironment favorable of the process of neoplastic transformation via paracrine and autocrine compositions [63, 64]. The estrogen-ER complex is transcriptional, which stimulates cell cycle regulating genes and anti-apoptotic pathway complexes [65]. Also, non-genomic estrogen-stimulated pathways through the membrane-bound receptors may promote growth of the tumour to resist therapeutic mechanisms. This mechanistic insight has supported the effectiveness of endocrine treatments as tamoxifen and aromatase inhibitors in the treatment of ER + breast cancer [65].

2.2.3 Other gynecologic and ovarian malignancies

Although a causal relationship between estrogen dominance and ovarian cancer is not as clear as that of endometrial and breast cancers, recent findings indicate that prolonged estrogenic stimulation could initiate and/or promote the onset of some subtypes of ovarian cancer, especially endometrioid and serous carcinoma [66, 67]. Estrogen receptors are expressed by ovarian epithelial cells, with varying expression levels and unopposed estrogen can promote a pro-inflammatory microenvironment which is growth supportive in the ovarian stroma and fallopian tubes, thought to be the source of most so-called "ovarian" carcinomas [68]. Association to estrogen prominent conditions, such as cervical adenocarcinoma and some types of vulvar cancers, have been reported but the mechanism is not known. Interestingly, chronic exposure to high doses of exogenous estrosgen as in the case of diethylstilbestrol (DES) consumption has also been implicated in the development of clear cell carcinoma of the vagina and cervix, highlighting the carcinogenic potential of prolonged unopposed estrogen signaling throughout the female reproductive tract [68].

2.3 Importance of early detection of estrogen-driven tumors

2.3.1 Prognostic value

Tumors detected at an early stage are generally smaller, less invasive, and less likely to have metastasized; therefore, they are more amenable to surgical removal or localized therapy [69, 70]. In endometrial cancer, as an example, five-year survival rate at an early stage of the disease that is restricted to the uterus exceeds 90%. On the same note, early stages of ER + breast cancer can be easily treated using hormonal therapy and small operations detected through mammography [17, 71]. The patterns of cancer development biologically responsive to estrogens are, therefore, predictable in their pathophysiology, which can provide windows of opportunity of diagnosis prior to the complete transformation [5, 72]. The early molecular changes including the upregulation of ER, the abnormal proliferation markers or the imbalances of hormone metabolites have the potential to become valuable prognostic factors and can even enable clinicians to risk stratify the patients based on their risk and implement interventions proportionately [72].

2.3.2 Improved outcomes through early intervention

The clinical course of many estrogen-related tumors underscores the benefit of early intervention. In cases where estrogen-based tumors can be found before the development of more insidious characteristics such as; hormone independence, p53 loss, spread, or invasion, response to treatment through hormone based interventions is still strong [73]. Early treatment will prevent the development of more serious malignant growth or spread and therefore cut down the size of surgery and chances of systemic chemotherapy being necessary [73]. In the case of ER + breast cancers, predictive testing, including, Oncotype DX may also be performed to reveal the need to apply adjuvant chemotherapy. The early detection of atypia can be treated by progestins in endometrial hyperplasia or endometrial ablation done through hysteroscopy before the development of cancer [74]. In sum, early detection changes the paradigm of treatment to proactive management enhances the quality of life, cheaper health care expenditures and survival rates.

2.3.3 Limitations of serum and imaging biomarkers

Although it is obvious that early detection leads to increased survival rate of estrogen driven cancers, current diagnostic methods still have limitations in detecting these cancers at an early stage [75, 76]. Serum biomarkers such as CA-125, HE4, and estradiol levels often lack sufficient sensitivity and specificity for early-stage disease. Imaging, whether with ultrasound or magnetic resonance imaging (MRI), is helpful but typically is not helpful until enough anatomic changes have taken place to identify the tumor. Besides, estrogen-determined cancers do not necessarily initiate with apparent morphological alterations in early phases, which further makes detection by imaging even more troublesome [77–79]. The major challenge is in differentiating benign effects of estrogen with the malignant transformation. Biosensing technologies may also be valuable as an alternative as they allow for detection of molecular biosignatures, including ER activation, metabolites of estrogen, or oxidative DNA adducts. Creating new biosensors for real-time measurement of unopposed estrogen exposure and cellular response that have been validated could revolutionize early cancer detection, especially for people who are at high risk.

3 Estrogen receptors as biomolecular targets

3.1 Estrogen receptors (ER-alpha, and ER-beta, GPER)

The classical estrogen receptors (ER), ERα and ERβ are ligand-activated nuclear receptors which are encoded by estrogen receptors1 (ESR1) and estrogen receptors 2 (ESR2). They are members of the nuclear receptor superfamily, which are transcription-regulating factors that bind estrogens including 17β-estradiol [80]. The two classical nuclear estrogen receptors, ERα and ERβ are similar in their DNA-binding region (about 96% homology) but they differ in the ligand-binding and N-terminal regions attributing their peculiar transcriptional characteristics and ligand responsiveness [5]. ERα is highly expressed in reproductive tissues such as the uterus, mammary gland, ovary, and hypothalamic-pituitary axis, whereas ERβ has a broader and tissue-dependent distribution, including the ovary, prostate, lung, gastrointestinal tract, cardiovascular system, immune cells, and central nervous system [81]. A third type of estrogen receptor, G protein-coupled estrogen receptor (GPER, formerly GPR30, is a membrane-bound receptor that links faster (non-genomic) effects of estrogen and occurs in places like the heart, liver kidney, and brain [82]. GPER, or GPR30, is not a classical nuclear receptor. It is a seven-transmembrane G protein-coupled receptor that mediates rapid, non-genomic estrogen signaling through second messengers and kinase pathways [83].

Both ERα and ERβ bind to 17β-estradiol with high affinity, but the receptors can exhibit differential response depending on the structure of the ligand, the structure of the receptor, tissue context and co-regulators present. GPER binds E2 as well, but its pharmacology and apparent affinity is different from ERα and ERβ, and the reported affinity is also variable for various assay systems [84]. ERα is more strongly bound to E2 and some synthetically produced estrogens such as ethinylestradiol, whereas ERβ has selective affinity to phytoestrogens, such as genistein [85]. Conformational change within the ligand-binding region alters the binding affinity, and thus the recruitment of co-regulators and gene transcriptional response as well. Although GPER binds E2 with a low affinity than ERα and ERβ, it can perform an active part in the mechanisms of fast signal transduction [5]. The structure of GPER does not possess the classical nuclear

receptors domains, but uses its seven-transmembrane topology, common to G-protein-coupled receptors, to activate second messenger pathways such as cAMP and PI3K/AKT signaling upon receptor ligand activation [86].

The structure and affinity variability between the ER subtypes determines its specific physiological and pathological functions [86]. ERa is largely related with proliferative response and is the main growth stimulator in estrogen-directed tumors like ER positive breast cancer. ERβ is usually thought to be anti-proliferative and counter regulatory to ERα, however, its activity is tissue specific and varies according to the disease [5]. GPER is mediator of rapid, non-genomic estrogen signaling which works in the cell proliferation, migration, as well as, cell apoptosis namely to the system of cardiovascular and the immune system [5, 87]. The subtle differences in distribution and affinity of each subtype of the ER allow important information regarding the role of this receptor in cancer formation to be informed, as well as highlight the potential role of the ER as a molecular target in the broad realm of diagnostics and therapeutics. These receptor differences are of significance for the biosensing applications as ERα, ERβ and GPER show different ligand binding behaviour, cellular localisations, conformational responses and different downstream signalling, thus the receptor to be functionalised with nanoprobes needs to be in line with the biological read out, and with the target analyte (Fig. 3).

3.2 ER in estrogen driven tumors

Estrogen receptors and more so the ERα are considered as central components of estrogen-receptive cancerous diseases given their role in mediating gene transcription via genomic and non-genomic mechanisms [60]. ERα and ERβ engage in conformational changes, dimerize and move to the nucleus and bind to estrogen response elements (EREs), located in the promoter of target genes upon binding to estrogens. In this interaction, co-activators or co-repressors are recruited which regulate the transcription of cell cycle progression genes, proliferation, differentiation and survival genes [88]. Alternatively, ERs may exercise their regulatory action through Tethering to another

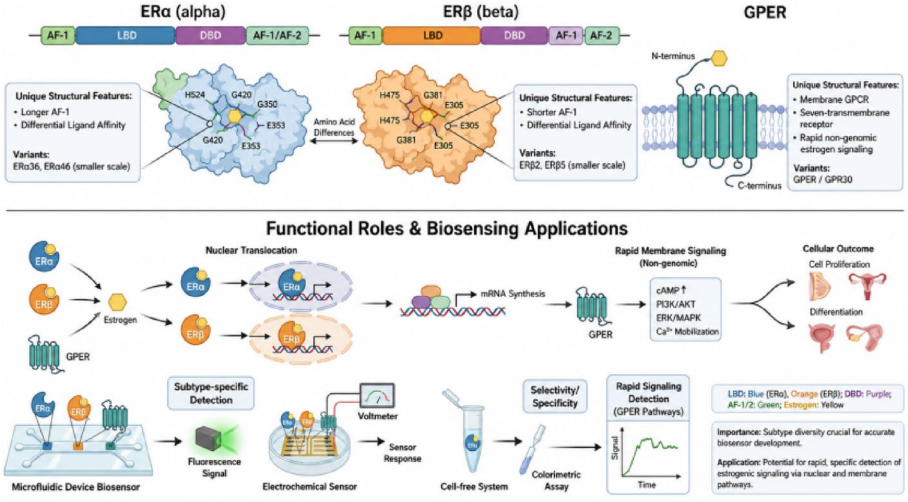


Fig. 3 Structural and functional diversity of estrogen receptor subtypes relevant to biosensing: ERα and ERβ represent classical nuclear estrogen receptors that primarily mediate genomic signaling, whereas GPER is a membrane-associated receptor involved in rapid non-genomic estrogen signaling. Inclusion of all three receptor subtypes highlights the importance of receptor-specific structure, signaling behavior, and biosensing strategy for accurate detection of estrogenic activity

transcription factor like AP-1 and SP-1. Concurrently, non-genomic pathways to activate signaling pathways such as MAPK, PI3K/AKT, and calcium mobilization involving GPER play roles in ligand dependent or independent cell proliferation and metastasis [88, 89].

About 70 percent of breast cancers are characterized by the overexpression of ERα, thus it is a linchpin biomarker of diagnosis, prognosis and therapy. In clinical practice, immunohistochemical assessment of ER status is used to classify tumors and guide endocrine therapy. Therapeutic strategies include selective estrogen receptor modulators such as tamoxifen, aromatase inhibitors such as anastrozole, letrozole, or exemestane, and selective estrogen receptor degraders such as fulvestrant or newer oral SERDs. These approaches reduce estrogen signaling either by blocking ER activity, decreasing estrogen synthesis, or promoting ER degradation [90]. In addition to breast cancer, ERs are involved in endometrial and ovarian cancer whereby estrogenic effect increases tumor growth [91, 92]. The presence, absence, or level of ER expression in tumors can assist in structural planning of therapy, defining drug response, and the speculation of the threat of a relapse of the infection. Targeting ERs therapeutically targets ERs have a goal of blocking either the activation of the receptor or the downstream signaling to prevent the role of tumor progression driven by estrogen [93].

Although ER-targeted therapies yielded good results, resistance can occur most commonly as a result of mutations on the receptor, alternative splicing, post-translational modifications or cross talk with other signaling pathways, e.g. HER2. Mutation of ERα ligand-binding domain (e.g. Y537S, D538G) may be able to impart constitutive activation of receptor leading to reduced efficacy of SERMs [5, 94]. Also, cross-regulation between ER and growth factor signalling makes the treatment option more complicated. In turn, the new treatment methods are under research, namely, selective estrogen receptor degraders (SERDs) which are ER antagonists that promote removal of the receptor, combination regimens that aim to block ER and co-activated oncogenic signaling. Recognition of the complicated nature of ER-mediated oncogenic signaling continues to play an essential role in establishing permanent therapy-based approaches in hormone-dependent malignancies [43].

3.3 ERs suitability to nanoprobe functionalization

Suitability of ERα and ERβ for nanoprobe functionalization is due not only to the presence of defined ligand-binding domains but also their capacity to sense estrogenic ligands, particularly 17β-estradiol, resulting in the specific interaction between ER and 17β-estradiol. Receptor-based recognition interfaces can be constructed by immobilizing either full-length receptors or isolated ligand-binding domains on nanomaterial surfaces by means of the use of recombinant full-length receptors or isolated ligand-binding domains. Key elements are receptor orientation, conformational stability to preserve the ligand binding pocket to maintain sensitivity and selectivity [17]. This specificity is especially helpful when low levels of estradiol or endocrine-disrupting compounds have to be detected in clinical samples or in environmental matrices. Besides, by using the ER-based nanoprobes, one can differentiate the presence of estrogenic and non-estrogenic analytes, which minimized false positives and increased the reliability of diagnostics, particularly those performed in terms of early cancer treatments [95].

The phenomenon of successful immobilization of estrogen receptors on different surfaces of nanomaterials, such as gold nanoparticle, quantum dots, carbon nanotubes, and graphene oxide, has been witnessed without the major loss of activity [17, 20]. Oriented immobilization procedures can maintain the structural integrity of their recognition elements and maintain binding capacity, including his tag/ni nta affinity coupling or covalent coupling using amine or thiol linkers [17]. These conjugated nanoprobes are understandable into optical, electrochemical, or colorimetric biosensors in order to allow real-time, label-free estrogen compounds detection. Moreover, the conformational shifts in ER upon ligand binding is itself a measurable detection event, and thus the changes in signal could be used as an output signal given by fluorescence resonance energy transfer (FRET) or impedance besides others depending on the sensing platform [17, 60].

The potential use of ER-functionalized nanoprobes in biosensing has promising future when it comes to non-invasive diagnostics and environmental monitoring [96]. The detection of unopposed estrogen in the body requires the use of an ER based biosensor in oncology to identify an early sign of the formation of hormone-mediated diseases like breast or endometrial cancer [97]. They are appealing models of point-of-care testing because they provide fast, sensitive and cost-effective analysis [98, 99]. Moreover, the ER-functionalized nanoprobes may be combined also with wearable or implantable biosensors to address hormonal changes in a continuous way and even provide new paradigms of personalized medicine [98, 100]. Estrogen receptors are versatile, specific, and adaptable biomolecular recognition elements, making them valuable components in next-generation biosensor design.

4 Nanoprobes in biosensing: fundamentals and functionalization

4.1 Nanoprobe-based biosensing principles

A nanoprobe is a nanoscale material or construct that senses a specific biological or chemical entity that produces a measurable product. In biosensing, nanoprobes are the main ingredient of the process of interaction with a specific analyte that causes a measurable indication [17]. Their diversity in small dimensions (1–100 nm), favorable surface-to-volume relationships and their designable surface chemistry make nanoprobes easy to interact at the molecular level with biological systems, which is exceptionally sensitive and specific [60]. There are four main categories of nanoprobes which are fluorescent nanoprobes, electrochemical nanoprobes, plasmonic (usually gold based) nanoprobes, and magnetic nanoprobes [72, 101–104]. The classes each use varying physicochemical mechanisms to sense the target molecules, whether hormone such as estrogen, enzyme, nucleic acid or cancer biomarkers.

Fluorescent nanoprobes are based on the effect of fluorescence resonance energy transfer (FRET) or direct fluorescence emission. These nanoprobes are usually fluorophore-labelled, i.e., they contain molecules with a propensity to absorb light of a particular longer wavelength and then to re-emitting it at an even longer wavelength, or they are made of naturally fluorescent nanomaterials, including quantum dots (QDs) [105]. The fluorescent signal is changed through conformational or electronic changes that occur on the binding of a target molecule [105]. This transformation has a relationship with the concentration of the analyte which can be quantitatively correlated. Fluorescent

nanoprobes constitute a flexible tool in cell visualization, live imaging, and the singlet detection of molecules in virtue of their high resolutions and sensitivity [105].

Nanoprobes based on electrochemical measurement depend on alterations to electrical characteristics (current, potential or impedance) as a result of the interaction between the nanoprobe and the analyte [60, 106, 107]. Such sensors are typically constructed in combination with conductive nanomaterials (e.g. carbon nanotube or gold nanoparticles) with biological recognition elements such as antibodies or estrogen receptors attached to them. Upon binding of target molecule, an electrochemical change is effected and is measurable [108]. The biosensors have the potential to be miniaturized, are low cost, and can work with the portable electronics therefore it is favorable to be used in point of care diagnostics. In addition, they can fulfill fast biomarker detection since they have high signal noise ratio together with real time feedback abilities [109, 110].

Plasmonic nanoprobes and magnetic nanoprobes have different but complementary action mechanisms. Plasmonic nanoprobes are commonly made of noble metals such as gold or silver and take advantage of the surface plasmon resonance (SPR) effect, i.e. collective motion of conduction electrons at the surface of a nanoparticle under illumination [60, 111]. A change in the local refractive index from binding of the target molecule leads to a red or blue shift of the plasmonic resonance peak which can be read out optically. Magnetic nanoprobes Iron oxide nanoparticles will react to an outside magnetic field and can be controlled or sensed with a magnetic resonance or using magneto-resistive measurement [112]. They find specific application in the target enrichment, separation, and non-invasive imaging. On balance, nanoprobe-based biosensing combines the most advanced form of nano technology with biomolecular recognition to produce high sensitivity, fast detection with a potential of multiplexing to achieve early diagnosis of diseases [17, 60]. In this review, the term 'nanoprobe' is used for engineered sensing constructs with at least one nanoscale component, typically within the 1–100 nm range. However, the analytical performance of ER-functionalized nanoprobes depends not only on particle size but also on surface chemistry, receptor orientation, receptor density, linker length, antifouling protection, and preservation of ER ligand-binding activity.

4.2 Estrogen receptor functionalization

The functionalization of nanoprobes means the chemical treatment of the nanoprobe surface to incorporate the biological recognition units (e.g. estrogen receptors (ERs)) that allow specific interaction with estrogen or estroid-like molecules [20, 22]. The effectiveness of any biosensor using nanoprobes lies directly on the swift and stable immobilization of these receptors without losses of their functionality. The determinant to be taken into account is that the functionalization strategies can be categorized broadly as covalent and non-covalent, each of which carries certain advantages and deficiencies according to the purpose of application and the nature of a nanoprobe [113, 114].

Covalent conjugation takes place as a stable chemical bond occurs between the functional groups on the nanoparticle surface and that of reactive complementary group on the estrogen receptors [115, 116]. Other techniques are carbodiimide chemistry (e.g., EDC/NHS conjugation) involving the conjugation of carboxyl and amine, thiol-maleimide chemistry, and click chemistry [117]. Covalent strategies provide strong immobilization of receptors, and thereby prevent desorption at physiological conditions

and increase the reliability of sensors [117]. However, extreme reaction conditions of reaction or incorrect orientation may distort the structure of the receptor diminishing the activity of ligand-binding [117]. Thus, the site-specific functionalisation-like utilising of engineered label on ERs- is gradually spreading to maintain bioactivity at the same time immobilising the firms firmly [118]. This should be therefore considered as a bioactivity preserving step and not merely as a surface attachment step, aside from the ER immobilization. Random covalent coupling may introduce conformational changes in the ERa and ERb ligand-binding domains involving lysine, cysteine or other residues near the functional domains, resulting in a partial denaturation or apparent decrease in affinity for 17β-estradiol and/or loss of conformational signaling upon ligand binding. Future studies of immobilized ER functionalized nanoprobes should provide proof of retained receptor activity after immobilization via E2-binding assays, radioligand or fluorescent ligand displacement assays, Surface Plasmon Resonance/ Bio-Layer Interferometry (SPR/BLI) kinetic analysis, Quartz Crystal Microbalance with Dissipation monitoring (QCM-D), thermal-shift assays, circular dichroism, FTIR or intrinsic fluorescence spectroscopy. The binding of ER to the surface of a nanoprobe should be confirmed by the ability to bind the nanoprobe to the ligand, and the ability to generate a signal from it.

Non-covalent strategies are based on less demanding interactions; e.g. electrostatic forces, hydrophobic interactions, or capture based on affinity. An example would be that the ERs could be adsorbed to the surface of nanoparticle by electrostatic interactions between the positively charged units and the negative ligands on the nanoprobe [119]. Otherwise, streptavidin–biotin reaction presents an excellent affinity and specificity in binding biotinylated ERs to streptavidin-conjugated nanoparticle [120]. Non-covalent approaches tend to require fewer steps and allows retention of the native conformation of the receptor, but has the drawback that they are less stable in conditions that simulate normal physiological conditions or high-shear stress, yielding a loss of adsorbed receptor, or variable signalling [121, 122].

One drawback of nanoprobes with ER is that the attached receptor is not necessarily natively active in binding its ligands. ERα and ERβ are conformation-sensitive proteins, and the ligand-binding domain of these may be partially denatured, sterically constrained or electronically altered upon binding to the surface of a nanoparticle [17, 122]. Covalent chemistries like EDC/NHS and thiol–maleimide coupling methods give greater surface stability but can also modify lysine, cysteine and other residues close to the functionally important part of the molecule at random, resulting in lower binding to 17β-estradiol or in a failure of conformational change upon ligand binding [17, 122].. Non-covalent adsorption, on the other hand, can provide better retention of native folding, but can be subject to receptor desorption, surface reorganization or signal drift in physiological buffer solutions. Thus, such functionalization should not only be retained by determining the attachment of the receptor, but also by apparent affinity and binding kinetics and the stability of the signals over time [17, 122]. The orientation of the receptor is particularly important since the small steroid ligands have to enter the pocket of the estrogen binding domain. Random amine coupling may lead to a mixture of receptor orientations in which some bind to the nanoparticle surface with the ligand binding domain oriented away, leading to a low sensitivity and poor reproducibility [123]. Batch-to-batch-variability may be minimized, and exposure of receptors may be optimized

by using oriented immobilization methods, such as His-tag/Ni–NTA, biotin–streptavidin, Fc-tag capture, SpyTag/SpyCatcher, or engineered terminal cysteine residues. Spacer arms e.g. PEG linkers should also be optimized as short spacers can create steric restrictions, while longer spacers can induce increased conformational flexibility and background signal. Future studies should specifically mention a method for controlling orientation; rather than just stating that ER was immobilized [123]. The linker lengths and linker orientation strategy should also be optimized experimentally. Short linkers can make the small steroid ligands less accessible to binding to the ER ligand-binding pocket and long or flexible linkers can allow for nonspecific movement of the small steroid ligand, background signal, and variability in the assay results. Thus, oriented immobilization should be compared to random immobilization, quantitatively by parameters such as surface receptor density, apparent Kd, signal to noise ratio, the response time, and intra-assay and inter-assay coefficience of variation. This would aid towards elucidating the available, selective and reproducibleness of the immobilized ER under biosensing circumstances [123]. Post-functionalization is also required in studies of stability on how better the receptors conformation and functional state is maintained as a function of time and under various conditions within the environment. Successful functionalization can commonly be validated by techniques e.g. circular dichroism (CD), surface plasmon resonance (SPR), ligand-binding assays [124].

Maintaining degree of biological activity is of special importance in the case of the sensors that measure unopposed estrogen, as it is crucial to detect the free, biologically active estrogen that is vital to proper diagnostics [19, 125]. Nanoprobes need to distinguish bioactive and inactive variations of estrogen. Hence surface engineering should not only inactivate receptors but also retain selective response to 17beta-estradiol or other estrogens of interest. These functionalized nanoprobes will thus be highly sensitive biosensors to identify very early oncogenic consequences of cancers of estrogenic origin, as an excellent weapon in precision medicine.

Post-functionalization validation needs to take place with structural and analytical assays. Radioligand/fluorescent ligand-binding assays, SPR, BLI, QCM-D, and thermal-shift assays are used for the quantification of surface loading and binding kinetics, with structural retention being assessed by circular dichroism and fluorescence spectroscopy, FTIR or thermal-shift assays. The following parameters should be covered in the analytical validation: limit of detection (LOD), limit of quantification (LOQ), linear dynamic range, response time, intra- and inter-assay coefficient of variation, storage stability, operational stability, and regeneration/reuse cycles. To translate to the clinic, recovery experiments in spiked serum, urine, and saliva should be reported, and compared with LC–MS/MS or validated immunoassay. Claims of good sensitivity or clinical utility without these quantitative parameters are preliminary. Successful biosensor construction depends on stable immobilization of estrogen receptors while preserving ligand-binding orientation and bioactivity (Fig. 4).

4.3 The category of used nanomaterials

The nanomaterial selected to comprise nanoprobes profoundly interferes with their physicochemical parameter, their signal transmission systems, and their efficiency of functionalization. Gold nanoparticle (AuNPs), quantum dot (QDs), magnetic nanoparticle (MNPs), carbon nanostructures (graphene and carbon nanotubes (CNTs) are a few

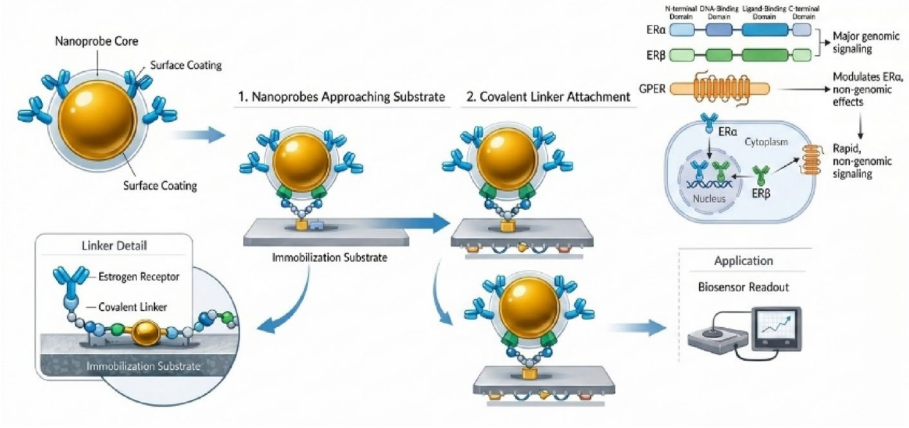


Fig. 4 Immobilization strategy for estrogen receptor-functionalized nanoprobes

of the nanomaterials that have been studied widely with regards to biosensing [126, 127]. All of these materials have individual benefits that are adapted to various sensing modalities and biological targets.

Gold nanoparticles have been one of the most commonly utilized nanomaterials applied in biosensing because of its wonderful biocompatibility and capability of functionalization, unique optic characteristics [128, 129]. AuNPs are powerful localized surface plasmon resonance (LSPR) labels, easily detecting molecular binding event-induced shifts in absorbance spectrum of scatter spectra [130, 131]. They are readily functionalized at their surfaces with thiol or amine linkers with subsequent conjugation of estrogen receptors or antibodies in a stable manner. Other than colorimetric and plasmonic, AuNPs can also be added to electrochemical sensor to augment the electron transfer which increases sensitivity. They have an ideal safety profile and their inert characteristics mean that they are ideal to be used in a clinical setting [19, 132].

Quantum dots are thin nanocryquinines of the semiconductor that glows with photoluminescence of tuneable sizes of brightness due to light excitation [133]. They are highly quantum yield, photostable and are capable of multiplexing, hence can be used to simultaneously detect multiple biomarkers [134]. When the QDs are functionalized with estrogen receptors they become highly sensitive and specific fluorescent biosensors to estrogen or estrogen-mimicking endocrine-disrupting chemicals [20]. But the worry of possible toxicity of QDs, at least that laden with heavy metals such as cadmium gives rise to the need to adhere to robust surface modification and cooking methods to make them safe to use [20].

Magnetic nanoparticle clusters with diameters less than 100 nm in size are used in a wide variety of applications such as, with few exceptions, biosensing and biomedical imaging with altered magnetic properties [135, 136]. Magnetic nanoparticles are most often produced using iron oxide (i.e. maghemite Fe_3O_4 or magnetite Fe_3O_4) [137]. They may be manipulated with external magnetic fields with targeted delivery, enrichment or separation of analytes. MNPs become selective receptors to estrogen when coated with estrogen receptor, allowing the isolation and purification of estrogen from complex biological matrices under the same conditions and later quantified [138]. They can also be combined with magneto-resistive sensors or serve as contrast agents in magnetic resonance imaging (magnetic resonance imaging MRI) and thus have both diagnostic and

therapeutic (theranostic) uses. They are suitable in vivo biosensing and point-of-care targeting because of their biocompatibility, and superparamagnetic nature [139, 140].

The carbon-based nanomaterials such as graphene, graphene oxide, or carbon nanotubes are gaining more researchers owing to their structural, electrical, and mechanical characteristics. Graphene sheets provide a wide area of immobilization of receptor molecules and good electrical conductivity of electrochemical analyses [141]. Due to their tubular selves, carbon nanotubes (CNTs) offer great aspect ratios and pathways of fast electron transfer [142, 143]. By functionalising these materials with estrogen receptors, one can sensitively and label-free detect estrogen molecules based on conductance change upon binding of the receptor-ligand. In addition, the vital plasticity of carbon-based nanostructures allows their embedding into wearable or implantable biosensors to monitor constant concentration of the hormones [143].

The effective design of biosensors for detecting unopposed estrogen relies on selecting suitable nanomaterials and optimizing their functionalization with estrogen receptors (ERα, ERβ)[19]. The common comparison of types of nanoprobes such as gold nanoparticles, quantum dots, magnetic nanoparticles, carbon nanotubes and structures of graphene, their detection methods, type of functionalization (covalent vs. non-covalent) and compatibility with estrogen receptor biology, is shown in Table 1. Performance parameters that are important like sensitivity, biocompatibility, multiplexing capability, and the ability to be incorporated internally in diagnostic devices are provided to depict how they can be used in early cancer detection. The table also foresees the stability and reusability of each system, which decides upon the build development of robust, point-of-care biosensors. It will be beneficial to view functionalization as more than merely the final step of surface conjugation to the nanoprobe, and more as a parameter which will determine the reliability, selectivity, and the translatability of the nanoprobes to the clinic in the context of estrogen receptor. Receptor immobilization by ERα/ERB can include effects on receptor folding, ligand binding pocket reach, steric receptor densities and long-term stability; protein adsorption, steroid crossreactivity, and matrix dependent changes in signals may be introduced by biological matrices like serum, urine, and saliva. Hence, certain structural, analytical and clinical validation criteria should be considered to evaluate ER-functionalized nanoprobes. Table 2 is a summary of the key parameters used in the functionalisation and validation processes to be observed to ensure reproducible, selective and clinically significant estrogen biosensing.

At present, most ER-functionalized nanoprobe platforms should be interpreted as analytically promising rather than clinically validated diagnostic tools. Claims of diagnostic accuracy should therefore be supported by quantitative performance data, including LOD, linear range, response time, recovery in serum/urine/saliva, intra- and inter-assay coefficients of variation, cross-reactivity against related steroids, and agreement with LC–MS/MS or validated immunoassays. Where prospective clinical studies are unavailable, the technology should be framed as having translational potential rather than proven clinical benefit. These standard conditions have been listed in Table 2**.** As shown in Fig. 5, different nanomaterial platforms can be functionalized with ERα or ERβ to convert selective estrogen–receptor binding events into measurable optical, magnetic, or electrical signals.

Over all, no one single nanoprobe platform is superior for ER-functionalized estrogen biosensing. Despite all their advantages (e.g. robust surface chemistry, easy read-out

by optical methods, and the point-of-care potential), gold nanoparticles are still prone to aggregation, protein corona and matrix dependent color change. The drawbacks of quantum dots are possible photophysical variations, autofluorescence of the matrix, and toxicity from the heavy metals; still, they possess high fluorescence intensity, photostability and the capability of multiplexing. The platforms based on carbon nanotubes and graphene are appealing for electrochemical and wearable sensors due to their high conductivity and surface area but are plagued by fouling, surface heterogeneity, dispersion, and reproducibly in each device. Magnetic nanoparticles can be used for enrichment and separation of analytes from complex biofluids but can be affected by receptor leaching, magnetic aggregation, and matrix effects which can confound the results and reduce the reproducibility. Thus, the choice of platform for future use should depend on the target application: AuNPs and paper-based systems for low-cost point-of-care optical detection; QDs and SERS for higher sensitivity optical detection; carbon-based materials for electrochemical miniaturized device; and magnetic nanoparticles for further enrichment of samples in complex matrices.

5 Estrogen detection using ER-functionalized nanoprobes

5.1 In Vitro detection systems

Estrogen detection through estrogen receptor (ER)-functionalized nanoprobes is a fast-moving field of biosensing technology [166, 167]. These are systems, which determine whether and how much estrogenic compounds are presented in controlled, cell-free conditions like laboratory buffers or in micro-fluidic assay wells [168]. Their functionality is determined by the strong affinity of ERs particularly ERα and ERβ to bind to the endogenous estrogens such as estradiol (E2) and also to the synthetic or environmental Xenoestrogen (e.g. bisphenol A, phytoestrogens). ER functionalization of the nanoprobes allows the specific and sensitive detection of nanoprobes in a pattern that mimics the biological system of recognition of estrogens [169, 170].

There are two main assay formats that predominate in the screening of ER-based estrogens, competitive and non-competitive binding assays [171]. The competition assays used in determining the amount of estrogen in the sample are conducted by providing a known amount of labeled estrogen with a limited number of ER binding sites which is competed by the unknown amount of estrogen in the sample [171]. There is negative correlation between the sample estrogen concentration and the intensity of signals [172]. Estradiol and the other estrogens are small molecules, and typically do not possess two distinct epitopes for classical sandwich type immunoassays. Typically, the classic sandwich format is not appropriate for the direct detection of estradiol unless engineered recognition systems are employed, or signal amplification strategies and indirect methods are used. Competitive displacement assays are better suited for ER-functionalized nanoprobes, and should be viewed as the predominant assay. In these assays a small amount of labelled estradiol or immobilized estrogen analogues is used that competes with free estradiol in the sample for a limited amount of ER binding site, resulting in a signal that is inversely proportional to the amount of estradiol present in the sample. Although there is a possibility of alternative non-competitive approaches, they should be identified as unbounded or conformational-change assays (for example SPR, impedance sensing, FRET-based receptor conformational reporting, aptamer-assisted sensing and molecularly imprinted polymer-assisted sensing or cell-based estrogen-response

Table 1 Comparative analysis of nanoprobes and functionalization strategies for estrogen receptor-based biosensing

No	Nanoprobe platform/material	Detection principle	ER functionalization strategy	Target analyte/sample matrix	LOD	Linear range	Response time	Selectivity/interference evaluation	Recovery in real samples (%)	Stability/reproducibility	Main advantages	Key limitations/translation concerns	References
1	Quantum dot-based fluorescent nanoprobes	Fluorescence emission or FRET signal change after ER–ligand interaction	Covalent coupling, EDC/NHS chemistry, or biotin–streptavidin attachment	E2 or estrogenic ligands in buffer, cells, serum, or environmental samples	NR	NR	NR	Should be tested against E1, E3, progesterone, testosterone, cortisol, phytoestrogens, and xenoestrogens	NR	Photostability should be reported; receptor activity after immobilization should be verified	High fluorescence intensity, multiplexing potential, real-time optical detection	Possible QD toxicity, photophysical variability, receptor denaturation, matrix autofluorescence	[144, 145]
2	Gold nanoparticle-based plasmonic/colorimetric nanoprobes	LSPR shift, absorbance change, or visible color change after analyte binding	Thiol–Au chemistry, PEG linker chemistry, or oriented receptor capture	E2 or estrogenic compounds in buffer, serum, urine, or saliva	NR	NR	NR	Cross-reactivity testing required against structurally similar steroids and serum proteins	NR	Colloidal stability and batch-to-batch AuNP size variation should be reported	Simple optical readout, label-free detection, strong surface chemistry, POC potential	Aggregation, nonspecific adsorption, protein corona formation, matrix-dependent color change	[72, 146]
3	Electrochemical AuNP/CNT/graphene nanoprobes	Current, potential, or impedance change following ER–estrogen binding	Covalent immobilization through carboxyl, amine, thiol, or linker-based chemistry	E2 in serum, saliva, urine, or buffer	NR	NR	NR	Should evaluate E1, E3, progesterone, testosterone, cortisol, cholesterol-derived steroids, and endocrine therapy compounds	NR	Intra-assay/inter-assay CV and electrode-to-electrode variability should be reported	High sensitivity, miniaturizable, compatible with portable and point-of-care devices	Electrode fouling, signal drift, receptor orientation variability, need for matrix-matched calibration	[60]
4	Magnetic nanoparticle-based probes, including Fe_3O_4 nanoprobes	Magnetic separation, enrichment, magnetoresistive readout, or MR signal change	Silane, dextran, carboxyl, or streptavidin–biotin surface chemistry	E2 or estrogenic compounds in complex biofluids or preconcentrated samples	NR	NR	NR	Should assess nonspecific adsorption from albumin, SHBG, globulins, and lipoproteins	NR	Magnetic stability, receptor leaching, regeneration cycles, and storage stability should be reported	Useful for analyte enrichment, separation from complex matrices, and possible theranostic integration	Receptor immobilization may alter binding activity; magnetic aggregation and matrix effects may reduce reproducibility	[147, 148]
5	Carbon nanotube-based ER nanoprobes	Conductance, resistance, or field-effect signal change after hormone binding	π–π stacking, carboxyl activation, covalent sidewall modification, or linker-assisted immobilization	E2 in buffer, saliva, serum, or wearable sensing formats	NR	NR	NR	Cross-reactivity and nonspecific adsorption should be tested under physiological ionic strength	NR	Device-to-device variability, baseline drift, and operational stability should be reported	High electrical conductivity, label-free detection, wearable sensor potential	Surface heterogeneity, fouling, poor dispersion, reproducibility challenges	[149, 150]
6	Graphene/graphene oxide ER nanoprobes	Electrochemical, impedance, or optical signal change	Covalent coupling, π–π interaction, PEGylated linker chemistry, or mixed surface functionalization	E2 or estrogen metabolites in buffer, serum, saliva, or urine	NR	NR	NR	Should include steroid cross-reactivity and serum interference studies	NR	Long-term signal retention and inter-batch reproducibility should be evaluated	Large surface area, high conductivity, strong signal amplification, real-time detection potential	Nonspecific adsorption, variable oxidation state, surface defects, signal drift	[151, 152]
7	ER-functionalized SERS nanoprobes	Raman spectral enhancement after estrogen binding near plasmonic nanostructures	ER immobilization on Au/Ag nanostars, nanorods, or roughened plasmonic substrates	Trace E2 or estrogenic compounds in buffer or biofluids	NR	NR	NR	Should confirm spectral specificity against E1, E3, steroid hormones, and serum components	NR	Substrate uniformity and spot-to-spot reproducibility should be reported	Ultra-sensitive detection and molecular fingerprinting	Requires reproducible SERS substrates; possible matrix interference and complex instrumentation	[153, 154]

Table 1 (continued)

No	Nanoprobe platform/material	Detection principle	ER functionalization strategy	Target analyte/sample matrix	LOD	Linear range	Response time	Selectivity/interference evaluation	Recovery in real samples (%)	Stability/reproducibility	Main advantages	Key limitations/translation concerns	References
8	ER-functionalized SPR sensor chips	Label-free refractive index change during ER–ligand binding	ER immobilization on gold-coated chips using amine coupling, thiol chemistry, His-tag/Ni–NTA, or biotin–streptavidin	E2, estrogenic ligands, or binding kinetics in buffer and diluted biofluids	NR	NR	Real-time	Should report kinetic discrimination among E2, E1, E3, xenoestrogens, and endocrine-disrupting compounds	NR	Regeneration efficiency, baseline drift, and repeated-cycle binding activity should be evaluated	Real-time kinetic analysis, label-free detection, direct Kd estimation	Requires specialized instrumentation; receptor orientation and regeneration may affect accuracy	[155, 156]
9	Paper-based or lateral-flow ER nanoprobes	Colorimetric, fluorescence, or capillary-flow signal readout	ER-coated AuNPs, latex particles, or cellulose-supported nanoprobes	E2 in urine, saliva, or field samples	NR	NR	NR	Should test false positives from steroid hormones and matrix components	NR	Shelf-life, humidity tolerance, and lot-to-lot reproducibility should be reported	Low-cost, disposable, rapid, suitable for field or home testing	Lower quantitative precision, matrix effects, limited sensitivity compared with laboratory platforms	[157–159]
10	Microfluidic/lab-on-chip ER nanoprobe systems	Integrated optical, electrochemical, or multiplexed detection	Patterned ER-functionalized nanomaterials in microchannels or sensor arrays	E2, E1, E3, or multiplex hormone profiles in small-volume biofluids	NR	NR	NR	Should include multiplex selectivity and matrix-matched calibration	NR	Chip-to-chip reproducibility, flow stability, and long-term storage should be reported	Low sample volume, automation, multiplexing, POC compatibility	Fabrication complexity, standardization, need for clinical validation	[43, 160]
11	Wearable or flexible ER nanoprobe sensors	Continuous electrochemical, impedance, or optical readout	ER-functionalized flexible electrodes, films, or hydrogel/nanomaterial interfaces	Sweat, saliva, or interstitial fluid hormone monitoring	NR	NR	Continuous/real-time; exact response time NR	Should test sweat/saliva interferents, pH variation, salts, and structurally related steroids	NR	Mechanical stability, bending tolerance, biofouling, and long-term drift should be reported	Continuous monitoring, wireless integration, personalized hormone tracking	Early-stage technology; calibration, biofluid correlation, and clinical interpretation remain unresolved	[161, 162]
12	Hybrid dual-mode ER nanoprobes	Combined optical and electrochemical, or magnetic and optical readout	Multicomponent ER-functionalized nanomaterials with linker or affinity-based immobilization	E2 or estrogenic compounds in biofluids or diagnostic platforms	NR	NR	NR	Orthogonal readouts should confirm selectivity against related steroids and matrix interferents	NR	Agreement between readout modes, reproducibility, and storage stability should be reported	Improved confidence through confirmatory dual-signal detection	Greater fabrication complexity and need for standardized calibration	[163–165]

ER estrogen receptor, *ERα* estrogen receptor alpha, *ERβ* estrogen receptor beta, *E1* estrone, *E2* 17β-estradiol, *E3* estriol, *LOD* limit of detection, *FRET* Förster resonance energy transfer, *LSPR* localized surface plasmon resonance, *SPR* surface plasmon resonance, *SERS* surface-enhanced Raman scattering, *AuNPs* gold nanoparticles, *CNTs* carbon nanotubes, *GO* graphene oxide, *QDs* quantum dots, Fe_3O_4 iron oxide nanoparticles, *SHBG* sex hormone-binding globulin, *CV* coefficient of variation, *POC* point-of-care, *NR* not reported

Table 2 Critical functionalization and validation parameters for ER-functionalized nanoprobes

Parameter	Why it matters	Recommended evaluation
ER surface density	Insufficient receptor loading reduces sensitivity, while excessive loading may cause steric crowding, receptor aggregation, or reduced ligand access	Quantify receptor loading using protein assays, fluorescence labeling, XPS, SPR, QCM-D, or BCA/Bradford assays before and after immobilization
Receptor orientation	The estrogen-binding pocket must remain exposed for effective recognition of 17β-estradiol and other estrogenic ligands. Random immobilization may block the ligand-binding domain	Compare oriented immobilization strategies such as His-tag/Ni–NTA, biotin–streptavidin, Fc-tag capture, SpyTag/SpyCatcher, or engineered terminal cysteine coupling
Retained binding activity	Immobilization may denature ERα/ERβ or reduce ligand affinity, causing loss of analytical sensitivity and unreliable signal generation	Perform E2-binding assays, SPR/BLI kinetic analysis, fluorescence ligand-binding assays, or radioligand displacement assays; compare apparent Kd values with free receptor
Stability after immobilization	ERs may lose conformation or detach from the nanoprobe during storage, repeated use, or exposure to physiological pH, salt, and temperature	Assess storage stability, thermal stability, pH/ionic-strength tolerance, repeated-use cycles, regeneration performance, and signal retention over time
Serum protein interference	Albumin, sex hormone-binding globulin, globulins, and lipoproteins can form a protein corona, mask ER binding sites, sequester estradiol, or cause nonspecific signal changes	Test in diluted and undiluted serum; use anti-fouling coatings such as PEG, zwitterionic polymers, BSA blocking, or mixed self-assembled monolayers; perform matrix-matched calibration
Selectivity against other steroid hormones	ER-based recognition is biologically relevant but not fully exclusive; structurally related steroids or estrogen metabolites may cause cross-reactivity or matrix effects	Evaluate cross-reactivity using E2, E1, E3, progesterone, testosterone, cortisol, cholesterol-derived steroids, phytoestrogens, xenoestrogens, and endocrine therapy compounds
Analytical sensitivity	Detection of bioavailable estrogen requires sensitivity within clinically or biologically relevant concentration ranges	Report LOD, LOQ, signal-to-noise ratio, calibration slope, response time, and minimum detectable concentration in buffer and biological matrices
Linear dynamic range	A useful biosensor must quantify estrogen across physiological and pathological concentration ranges without signal saturation	Generate calibration curves across multiple E2 concentrations and report the linear range, regression equation, R^2 value, and upper/lower quantification limits
Reproducibility and precision	Batch-to-batch variability in nanoparticle synthesis and ER immobilization can reduce reliability and limit clinical translation	Report intra-assay and inter-assay coefficient of variation, batch-to-batch variation, replicate number, and inter-operator or inter-laboratory reproducibility
Real sample recovery	Performance in buffer does not guarantee accuracy in serum, urine, saliva, or tissue-derived samples	Perform spike-and-recovery experiments in serum, urine, saliva, or relevant biofluids; report percentage recovery, dilution linearity, and matrix effects
Accuracy versus reference methods	Clinical adoption requires comparison with established analytical methods	Compare results with LC–MS/MS, validated ELISA, chemiluminescent immunoassays, or other accepted hormone assays using correlation analysis and Bland–Altman plots
Regeneration and reusability	Reusable sensors must retain ER activity after ligand removal and repeated measurement cycles	Test regeneration buffers, number of reusable cycles, percentage signal recovery, baseline drift, and retained binding capacity after repeated E2 exposure

Table 2 (continued)

Parameter	Why it matters	Recommended evaluation
Anti-fouling performance	Nonspecific adsorption can increase background signal and reduce selectivity, especially in serum-rich matrices	Compare uncoated and coated nanoprobes using PEGylation, zwitterionic coatings, BSA/casein blocking, or hydrophilic polymer brushes; report nonspecific binding reduction
Clinical applicability	A clinically useful ER-functionalized nanoprobe must be stable, accurate, selective, and compatible with point-of-care or laboratory workflows	Validate assay performance in clinically relevant sample numbers, include healthy and high-risk groups, define diagnostic cutoffs, and report sensitivity, specificity, ROC-AUC, and predictive values

ER estrogen receptor, *ERα* estrogen receptor alpha, *ERβ* estrogen receptor beta, *E1* estrone, *E2* 17β-estradiol, *E3* estriol, *LOD* limit of detection, *LOQ* limit of quantification, *Kd* equilibrium dissociation constant, *SPR* surface plasmon resonance, *BLI* bio-layer interferometry, *QCM-D* quartz crystal microbalance with dissipation monitoring, *XPS* X-ray photoelectron spectroscopy, *FTIR* fourier-transform infrared spectroscopy, *LC–MS/MS* liquid chromatography–tandem mass spectrometry, *ELISA* enzyme-linked immunosorbent assay, *ROC-AUC* area under the receiver operating characteristic curve, *CV* coefficient of variation, *PEG* polyethylene glycol, *BSA* Bovine serum albumin, *MIP* molecularly imprinted polymer, *Ni–NTA* nickel–nitrilotriacetic acid, *His-tag* histidine tag, *Fc-tag* fragment crystallizable tag, *SHBG* sex hormone-binding globulin, *POC* point-of-care, R^2 Coefficient of determination, *SAM* self-assembled monolayer

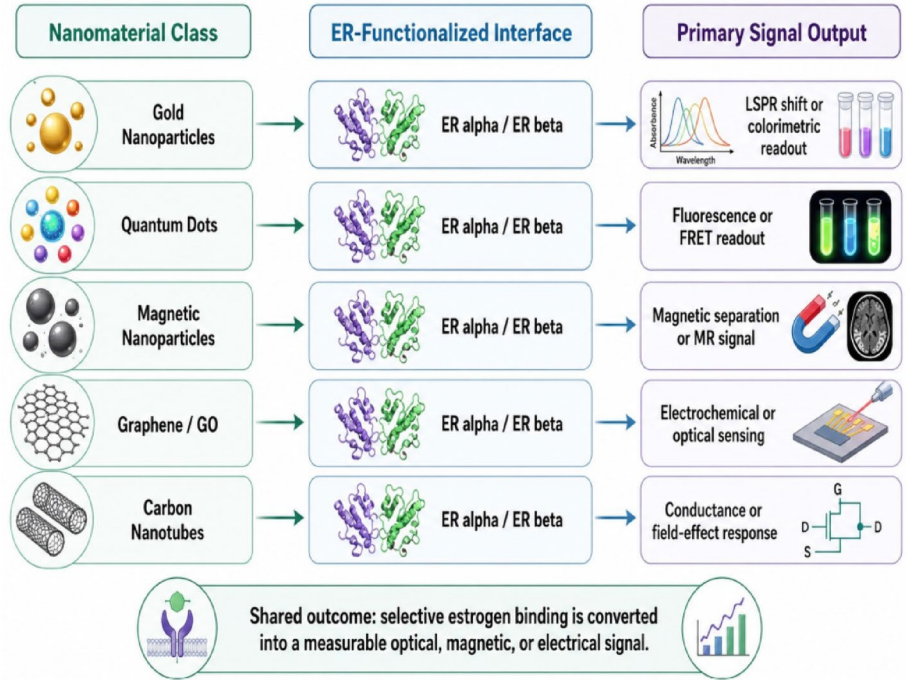


Fig. 5 Nanomaterial platforms for estrogen receptor-based biosensing: Schematic representation of the main classes of nanomaterials that have been employed for ER-functionalized estrogen biosensors: gold nanoparticles, Quantum Dots, magnetic nanoparticles, graphene oxide, and carbon nanotubes. Each platform has associated ERα/ERβ-recognition interfaces, and generates a unique signal output, including but not limited to LSPR colorimetric shifts, fluorescence/FRET readout, magnetic separation or MR signal, electrochemical/optical sensing, or conductance/field-effect response

reporter assays) rather than sandwich assays [172]. These techniques commonly use nanomaterials like gold nanoparticle, quantum dots and carbon nanotubes to provide signal amplification, which enhances the performance of the assay.

These important assay parameters include sensitivity and specificity. The sensitivity is the measurement of the capability to measure up low concentrations of estrogen, which can be in femtomolar (fM) to nanomolar (nM)[173–175]. Specificity on the other hand, refers to the capacity of the probe to discriminate elements of estrogen and those molecules of similar structure [175]. Raising both parameters has been achieved, through

the functionalization of nanoprobes with recombinant or purified ERs. Also, once Again, when aptamers or molecularly imprinted polymers are integrated with ERs, target specificity has been further optimized. Multiplex detection and automation, as well as throughput and reproducibility have been enhanced with the improved microfluidics and lab-on-chip systems [176–178].

Nonetheless, with these fulfilled goals, there are issues related to assuring the stability of the ER-functionalized nanoprobes, avoiding the drift in the signal, and preserving the receptor conformation in vitro [179, 180]. However, this method is an essential basis of establishing transportable biosensors, and a screening of environment/pharmaceutical estrogens in an efficient manner. Observations made in such cell-free systems have a direct translation to more biologically significant systems as well as to diagnostic. Selectivity is especially significant, since ER based recognition also pertains to physiologically relevant recognition that does not, however, necessarily exclude 17β-estradiol. ERs or therapeutic drugs and endocrine disrupting compounds (EDCs) which have a similar structure to endogenous steroids can interact with the ERs or influence the output signal of the biosensor. Thus, nanoprobe platforms functionalized with ER should be evaluated for their ability to bind and produce a response to estrone, estriol, progesterone, testosterone, cortisol, cholesterol-derived steroids, phytoestrogens, xenoestrogens, selective estrogen receptor modulators, aromatase inhibitors, and other endocrine-active compounds. Any cross reactivity should be quantitatively expressed as the percentage signal response to E2 at clinically relevant concentration.

5.2 Tissue and cellular imaging

ER-functionalized nanoprobes have been developed as an extremely useful utility in cellular and tissue imaging, especially in the context of sensitive estrogen receptor (ER) and its location in cancerous estrogen receptor-positive (ER +) cells [43, 181]. Molecular recognition tools are encompassed in these tools which integrate procedures in imaging technologies (fluorescence, confocal microscopy, or, photoacoustic imaging). Nanoparticles can be functionalized with ER ligands or antibodies and preferentially target ER showing cells, providing molecularly precise and high-resolution imaging [182, 183].

With regard to such ER-positive cancers as some breast, endometrial and ovarian types, the nanoprobes are able to realize tissue section or live cell culture distribution and density of ERs [184, 185]. As an example, fluorescently tagged gold nanoparticle conjugated with ER ligands can be internalized into ER positive cancer cells and can be visualized in real time. This gives an understanding of the dynamics of estrogen signaling dynamics and information on the pharmacodynamics of estrogen-therapiies such as tamoxifen [186, 187]. The possibility of real-time observation of estrogen interactions in real living cells has been enabled with the use of nanoprobe using quantum dot and upconversion nanoparticles, which possessed high signal-to-noise ratio and resistance to photobleaching [188–190].

The recently emerging trend towards combining the immunofluorescence approach with the biosensing response is encouraging, as an ER-functionalized probe could be applied not only to label the target cells but also to measure the estrogenic activity [191, 192]. As an example, nanoprobes conjugated with antibodies directed to ER would enable detection of levels of estrogen and intracellular localization of receptor to be visualized at the same time [193, 194]. When applied in the biosensing-imaging fusion

systems, a shift in fluorescence intensity or wavelength may occur upon ligand binding, which would be a dynamic property in indicating receptor engagement and subsequent signaling processes [193, 195]. This may be further elaborated with the use of Förster Resonance Energy Transfer (FRET) based nanosystems, which permits sub cellular observation of molecular interactions [196, 197].

ER-functionalized probes are used not only to diagnose but also to administer a therapeutic load. Chemotherapeutics or siRNA can be loaded into nanoparticles and guided to the ER + cells using nanop article DOT, which reduces the off-target and treatment toxic effects [198–201]. Yet, among the limitations, one can count possible toxicity of nanoprobes, non-specific uptake of nanoprobes in other parts of the body, and disparity in expression of receptors across tumors or among the population [202]. However, use of ER-functionalized nanoprobes in imaging can be considered a diagnostic and therapeutic approaches combination with a valuable promise in the realm of precision oncology.

5.3 Serum, urinary, salivary biofluids detection

Analysis of estrogen in biofluids like serum, urine, and saliva entails a set of challenges that is unique because there are a lot of proteins, salts, and other small molecules that may lend interference with the accuracy of the assay [203, 204]. However, ER-functionalized nanoprobes are in development to curb these obstacles, so as to perform non-invasive, sensitive, estrogen detection, which can be optimal in diagnosis and monitoring [205]. These methods play an important role in the early diagnosis of cancer and the endocrinology hormonal profiling [43].

Non-specific binding in which components of the nanoprobe could interact with non-targeted molecules and cause background signals or low sensitivity is the biggest problem with biofluid analysis [206]. To solve this problem nanoprobe may be engineered with an anti-foulant coating to prevent protein adsorption usually polyethylene glycol (PEG) or zwitterionic materials [207]. Furthermore, the binding of the probes to estrogen is selective due to the use of ER specific ligands or monoclonal antibodies which are used to assure that the probes would even bind to estrogen in extremely complex fluid conditions [208]. Serum is a major problem with matrix interference as it is rich in protein and this may alter nanoprobe stability as well as receptor conformation [209]. In contrast, urine and saliva contain less of the proteins but might have different amount of the estrogen metabolites [43]. In order to enhance performance, optional pre-treatment procedures of filtration, centrifugation or addition of microfluidic separation channels can separate the estrogen in other fluid constituents. Also, to be able to recognize the targets and decrease the false positives, it is possible to attach molecularly imprinted polymers (MIPs) onto the nanoprobes [210]. For clinical biofluid analysis, the nonspecific adsorption and matrix interference need to be validated more thoroughly. The various components of serum such as proteins (albumin or sex hormone binding globulin), globulins, lipids (lipoproteins), salts and metabolites can assemble around nanoprobes to form the 'protein corona', bind ER sites anaesthetic, or produce non-specific background signals. For this reason, evaluation of ER-targeted nanoprobes should be carried out in both the diluted and undiluted serum, urine and saliva matrices using matrix matched calibration. The effect of the anti-fouling strategy on these four parameters (reduction of the nonspecific adsorption to protein, percentage recovery, signal drift, and precision of the assay) should be quantitatively compared for strategies including PEGylation,

zwitterionic coatings, blocking with BSA or casein, use of hydrophilic polymer brushes, or mixed self-assembled monolayers (SAMs).

Improved portable and point-of-care (POC) platforms have greatly increased the applicability of ER-functionalized nanoprobes into the real world. Such devices frequently combine colorimetric molecular detection or electrochemical detection modalities which are readable using the smartphone-based apps or handheld readers. PADs (paper-based analytical devices) covered with ER-coated nanoparticles are cheap and can be disposable field tests or home diagnostics [211–214]. Additionally, lateral flow assays (LFA) biosensors have been constructed in detecting estrogen in urine and saliva which provides results in a matter of minutes. The introduction of wearable biosensors that allow monitoring estrogen continuously is another innovation that has a lot of potential to change fertility tracking, hormone replacement therapy and cancer monitoring [215]. Such systems can combine ER-functionalized nanomaterials, bendable electronics and wireless communications packages, although they do not yet have reached significant development.

As showed in Table 3, there is a notable comparative analysis of the different estrogen detection platforms based on the estrogen receptor (ER)- functionalized nanoprobes according to the three major fields in which it is to be utilized and which includes the in vitro systems, cellular and tissue imaging and, bio fluid based diagnostics. Within this comparative architecture, it is evident that with nanotechnology and receptor engineering, estrogen detection has changed and shifted at the most basic level of immunoassays to exquisitely sensitive, specific, and (in many cases) miniaturized bioresponse modalities. The platform is described according to the principle of detection, the type of nanomaterials implemented, the benefits of the device in comparison to the previous or conventional system, the analytical sensitivity (limit of detection, LOD), the selectivity towards the estrogen, than the related substances, as well as its application in clinical, research, or field settings, as shown in Table 3. Remarkably, the electrochemical sensors, FRET-based imaging probes, and surface-enhanced Raman scattering (SERS) technologies continue showing exceptional sensitivity, with detection limits of at least the picomolar to femtomolar range, which serves early-stage reactions to disorders and malignancies of hormone-related illnesses and estrogen-induced malignancies. In addition, other inventions include wearable bio sensors, paper-based and lateral flow assays and there has been a trend of increased shift to portable, point of care and a patient-friendly estrogen monitoring systems and especially in foods like saliva or sweat. Conversely, high-resolution imaging probes (tissue-targeted immunofluorescence imaging and photoacoustic imaging) may promote theranostic (diagnostic and therapeutic) applications in cancer. As shown in Fig. 6, ER-functionalized nanoprobes can support non-invasive estrogen monitoring by detecting estrogenic analytes in accessible biofluids and converting receptor–ligand interactions into measurable diagnostic signals.

6 Signal transduction and data readout strategies in functionalized nanoprobes for early cancer detection

Efficient development of biosensing into cancer diagnostics is highly demanding of signal transduction pathways and signal read out methods embedded on the nanoprobe platform [255]. Signal transduction describes how the recognition of a molecule happens and how this happening is transferred to a measurable signal [256]. These signs

may be optical, electrical or mechanical by the transducer being used. The determination of the readout strategy is vital to high sensitivity and specificity in addition to being compatible with real-time or in situ analysis [257, 258]. This section discusses four major categories of detection technologies applied on biosensing systems of ER-functionalized nanoprobe in early cancer detection.

6.1 Fluorescent and luminescent signaling

Among the most common optical modes of transduction in biosensors are on the one hand fluorescent and on the other hand luminescent signaling that boasts high sensitivity and ability to integrate into imaging platforms [259, 260]. The frequently applied mechanism is Förster Resonance Energy Transfer (FRET) which takes advantage of non-radiative energy transfer between a donor fluorophore and an acceptor due to proximity [261]. FRET in nanoprobe biosensors can be utilized to detect conformational shifts of ER-functionalized nanomaterials in combination with estrogen or the binding of estrogen to an ER functionalized nanomaterial [262]. As an illustration, quantum dots (QDs) semiconductor nanocrystals with tunable (bi)-emission are used, because of high quantum-yield, photostability, and multiplexing potential.

ER-conjugated QDs have the capability of emitting fluorescent signal when prematurely bound to estrogen or other metabolites of estrogen and extremely sensitive and spatially localized detection can be carried out [263]. Fluorescence-based methods are also advantageous because they often provide multiplexed detection which enables the possibility to analyse multiple hormone-receptor interactions at once [156, 264]. It is particularly useful in cancer diagnosis when there may be co-existence of numerous biomarkers. Moreover, the low-background autofluorescence in time-resolved luminescence applications like lanthanide-based probe-based methods and also leads to the improved precision of detection of biological complex samples [265].

6.2 Biosensing used in electrochemical sensors

The biosensors use the electrical signals predicted by biochemical interaction; electrochemical biosensors are becoming popular because of their small size, cheap prices, and sensitivity [266, 267]. In ER-enzymatically modified electrochemical biosensors, the events of binding estrogen involves current, potential or impedance alterations. ER-decorated nanomaterials including gold nanoparticles (AuNPs), graphene or carbon nanotubes (CNTs) can be attached on electrodes to improve surface area and electron transfer [268]. The redox reaction attributed with ligand binding is detected through Amperometric sensing when current changes. As an example, hormone binding may induce enzymatic or redox reaction that can produce current measurable [269]. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) voltammetric techniques can be used to carry out fine profiling of the electrochemical changes associated with hormone-receptor interactions [270, 271]. This strategy can be applied to point-of-care (POC) diagnostics because there is a high specificity of ER-nanoprobe alteration due to which the specific signal is produced only in complex biological media, e.g., serum or saliva [272].

Table 3 Comparative analysis of ER-functionalized nanoprobe platforms for estrogen detection across *In* vitro, imaging, and biofluid applications

No	Platform type	Detection principle	Nanomaterials/recognition system	Reported analytical performance	Tested sample matrix	Selectivity/cross-reactivity evaluation	Stability/reproducibility evidence	Clinical validation status	References
1	Competitive ER-binding assay	Free estrogen competes with labeled estrogen or immobilized estrogen analogue for limited ER-binding sites	ERα/ERβ, labeled E2 analogue, AuNPs, fluorophores, or enzyme labels	LOD and linear range should be stated only if reported; NR if not available	Usually buffer; sometimes diluted serum or urine	Should include E1, E2, E3, progesterone, testosterone, cortisol, phytoestrogens, and xenoestrogens	Often limited; receptor activity after immobilization should be verified	Mostly preclinical or analytical	[216, 217]
2	Electrochemical ER-functionalized sensor	Current, potential, or impedance change after ER–estrogen binding	CNTs, graphene, AuNPs, AgNPs, ERα/ERβ-modified electrodes	Some studies report pM-range LODs, but values must be verified in the original studies	Buffer, serum, saliva, or urine, depending on study	Cross-reactivity with E1, E3, progesterone, cortisol, and other steroids should be mandatory	Requires electrode-to-electrode CV, inter-assay CV, and signal drift testing	Limited clinical validation	[218, 219]
3	Fluorescent/FRET biosensor	Fluorescence change caused by ligand binding, receptor conformational change, or displacement	QDs, fluorophore-labeled estrogen analogues, ERα/ERβ	fM–pM LODs should be accepted only when supported by calibration, blank statistics, and replicate testing	Often buffer or environmental samples; fewer validated biofluid studies	Must distinguish E2 from E1, E3, xenoestrogens, and fluorescent interferents	Photostability and receptor stability should be reported	Mainly experimental	[220, 221]
4	SPR-based ER sensor	Label-free refractive-index shift during estrogen–ER binding	Gold SPR chip functionalized with ER or estrogen analogue	Usually real-time kinetic data; LOD and Kd should be reported separately	Mostly buffer or diluted biological samples	Should evaluate structurally similar estrogens and endocrine-active compounds	Regeneration cycles, baseline drift, and receptor activity should be reported	Preclinical analytical validation	[222, 223]
5	Colorimetric AuNP nanosensor	Visible color or absorbance shift after binding-induced aggregation or surface change	AuNPs functionalized with ER, aptamer, antibody, or estrogen analogue	Often nM–pM range; should be reported with linear range and response time	Buffer, urine, saliva, or diluted serum	Susceptible to matrix-induced aggregation; steroid cross-reactivity required	Colloidal stability and batch reproducibility required	Limited	[224, 225]
6	Aptamer- or MIP-assisted estrogen sensor	Ligand binding or displacement using synthetic recognition elements	Estrogen aptamers, MIPs, AuNPs, graphene, QDs	LOD varies widely; must be linked to assay format and matrix	Buffer and selected real samples	Should compare E2 with E1, E3, progesterone, cortisol, and endocrine disruptors	Generally more stable than protein ER systems, but imprinting/aptamer batch variability remains	Mostly analytical	[226, 227]

Table 3 (continued)

No	Platform type	Detection principle	Nanomaterials/recognition system	Reported analytical performance	Tested sample matrix	Selectivity/cross-reactivity evaluation	Stability/reproducibility evidence	Clinical validation status	References
7	Microfluidic ER-nanoprobe platform	Integrated sample handling with optical or electrochemical readout	ER-QD hybrids, ER-AuNPs, graphene electrodes, PDMS chips	LOD, response time, and sample volume should be reported; NR if unavailable	Small-volume serum, urine, saliva, or buffer	Matrix-matched calibration and multiplex steroid selectivity required	Chip-to-chip reproducibility and flow-dependent variability should be reported	Early-stage translational	[43, 228, 229]
8	Imaging-based FRET or fluorescence probe	Imaging of receptor localization, ligand binding, or estrogenic activity	ERα/ERβ probes, QDs, fluorophores, upconversion nanoparticles	LOD often not applicable unless quantitative calibration is performed	Cells, tissue sections, or animal models	Should assess nonspecific uptake and ER-negative controls	Photobleaching, probe stability, and cell-to-cell variability should be reported	Research use	[43, 229]
9	Photoacoustic or magnetic imaging probe	Optical absorption or magnetic contrast linked to ER-targeted accumulation	Iron oxide, Au nanostructures, ER-targeted conjugates	Quantitative LOD often not applicable	Cells, tissues, or animal models	Requires ER-positive and ER-negative controls	Long-term probe stability and biodistribution should be assessed	Preclinical	[230, 231]
10	Fluorescence immunosensor	Fluorescent signal generated by antibody or receptor-mediated recognition	ER-targeted antibodies, fluorophores, nanocarriers	Reported LOD must be verified against matrix and calibration design	Tissue, serum, or cell samples	Antibody specificity and steroid cross-reactivity should be separated analytically	Reagent stability and lot-to-lot reproducibility required	Variable	[232, 233]
11	Saliva or urine rapid assay	Competitive or displacement-based detection in non-invasive samples	Magnetic beads, AuNPs, latex particles, fluorophores, ER/aptamer/MIP	Usually pM–nM range; must include recovery and dilution linearity	Saliva or urine	Must address pH, salts, metabolites, E1/E3, progesterone, cortisol	Shelf-life and operational stability required	Limited	[234, 235]
12	Paper-based or lateral-flow device	Capillary-flow colorimetric or fluorescence readout	Cellulose, AuNPs, latex nanoparticles, ER or synthetic receptors	Often less sensitive than laboratory sensors; LOD should be verified in real samples	Urine, saliva, or field samples	False positives from matrix color, salts, and steroid analogues should be evaluated	Humidity, temperature, and storage stability required	Limited	[236, 237]
13	SERS-based estrogen sensor	Raman signal enhancement from estrogen near plasmonic nanostructures	Ag/Au nanostars, nanorods, ER-functionalized SERS substrates	Very low LODs may be reported, but must include substrate reproducibility and real-sample validation	Buffer or selected biofluids	Spectral specificity against E1, E3, progesterone, cortisol, and serum components required	Spot-to-spot and batch-to-batch SERS reproducibility required	Analytical/preclinical	[238–240]

Table 3 (continued)

No	Platform type	Detection principle	Nanomaterials/recognition system	Reported analytical performance	Tested sample matrix	Selectivity/cross-reactivity evaluation	Stability/reproducibility evidence	Clinical validation status	References
14	Dual-mode sensor	Two independent outputs, such as optical plus electrochemical	QDs, CNTs, AuNPs, graphene, ER or synthetic receptors	LOD should be reported for each mode separately	Buffer or biofluids	Orthogonal confirmation should reduce false positives	Agreement between modes and reproducibility should be reported	Early-stage	[34, 241]
15	Cell-based estrogen-response assay	Reporter-gene activation after functional estrogenic stimulation	ER-responsive cell lines with fluorescence/luminescence reporter	Measures estrogenic activity rather than only binding; LOD depends on reporter design	Cell culture, extracts, environmental samples	Detects functional estrogenicity but may respond to many ER agonists	Cell passage number, viability, and assay variability must be controlled	Research/toxicology use	[242, 243]
16	Multiplex nanoarray	Parallel detection of E2, estrogen metabolites, or related biomarkers	Barcode QDs, graphene arrays, ER/aptamer/MIP recognition elements	LOD and linear range must be reported per analyte	Buffer, serum, urine, or saliva	Cross-talk among analytes and cross-reactivity must be quantified	Array-to-array reproducibility required	Early-stage	[244, 245]
17	SERS-based detection	Raman signal enhancement from estrogen or reporter molecules near plasmonic nanostructures	Ag/Au nanostars, nanorods, roughened substrates, ER-functionalized SERS probes	Very low LODs may be reported, but must include substrate reproducibility and real-sample validation	Buffer or selected biofluids	Spectral specificity against E1, E3, progesterone, cortisol, and serum components required	Spot-to-spot and batch-to-batch SERS reproducibility required	Analytical/preclinical	[246, 247]
18	Dual-mode sensor	Two independent outputs, such as optical plus electrochemical signals	QDs, CNTs, AuNPs, graphene, ER, aptamer, or synthetic receptors	LOD should be reported for each readout mode separately	Buffer or biofluids	Orthogonal confirmation should reduce false positives, but must be experimentally shown	Agreement between modes and reproducibility should be reported	Early-stage	[248, 249]
19	ER-MIP sensor	Molecularly imprinted polymer mimics estrogen-binding cavity or ER-like recognition	MIP polymer films, electrodes, AuNPs, graphene, or hybrid materials	LOD varies; must include imprinting factor, linear range, and real-sample recovery	Buffer, pharmaceutical samples, urine, saliva, or serum extracts	Should test E1, E3, progesterone, cortisol, testosterone, and endocrine-disrupting chemicals	MIP film stability, regeneration, and batch reproducibility should be reported	Mostly analytical	[250, 251]

Table 3 (continued)

No	Platform type	Detection principle	Nanomaterials/recognition system	Reported analytical performance	Tested sample matrix	Selectivity/cross-reactivity evaluation	Stability/reproducibility evidence	Clinical validation status	References
20	Cell-based fluorescent/reporter assay	Reporter-gene activation after functional estrogenic stimulation through ER signaling	ER-responsive cell lines with fluorescence or luminescence reporter	Measures estrogenic activity rather than only binding; LOD depends on reporter design	Cell culture, extracts, environmental samples, or treated biofluids	Detects functional estrogenicity but may respond to many ER agonists	Cell passage number, viability, incubation time, and assay variability must be controlled	Research/toxicology use	[252, 253]
21	Multiplex nanoarray	Parallel detection of E2, estrogen metabolites, or related biomarkers	Barcode QDs, graphene arrays, ER/aptamer/MIP recognition elements, or multi-sensor chips	LOD, LOQ, and linear range must be reported separately for each analyte	Buffer, serum, urine, saliva, or tissue-derived samples	Cross-talk among analytes and cross-reactivity must be quantified	Array-to-array reproducibility and calibration stability required	Early-stage	[254]

While the results may demonstrate high analytical sensitivity for ER-functionalized nanoprobes, the fM-pM LODs need to be carefully understood and are only valid when determined with validated calibration protocols in clinically relevant matrices. Performance in buffer doesn't always reflect in serum, urine, or saliva, where the formation of protein corona, non-specific adsorption, hormone-binding proteins, salts, metabolites, and endogenous steroids can affect the availability of receptors and the signal. Future studies should thus be done in the presence of the specific matrix for which they are used, with spike and recovery, dilution linearity, cross reactivity with E1, E3, progesterone, cortexolone and xenoestrogens, ER immobilization stability, intra- and inter-assay variability, and comparison with LC–MS/MS or validated immunoassays prior to claiming clinical utility

ER estrogen receptor, *ERα* estrogen receptor alpha, *ERβ* estrogen receptor beta, *E1* estrone, *E2* 17β-estradiol, *E3* estriol, *LOD* limit of detection, *LOQ* limit of quantification, *Kd* dissociation constant, *CV* coefficient of variation, *QDs* quantum dots, *AuNPs* gold nanoparticles, *AgNPs* silver nanoparticles, *CNTs* carbon nanotubes, *MIP* molecularly imprinted polymer, *SPR* surface plasmon resonance, *SERS* surface-enhanced Raman scattering, *PDMS* polydimethylsiloxane, *POC* point-of-care, *NR* not reported

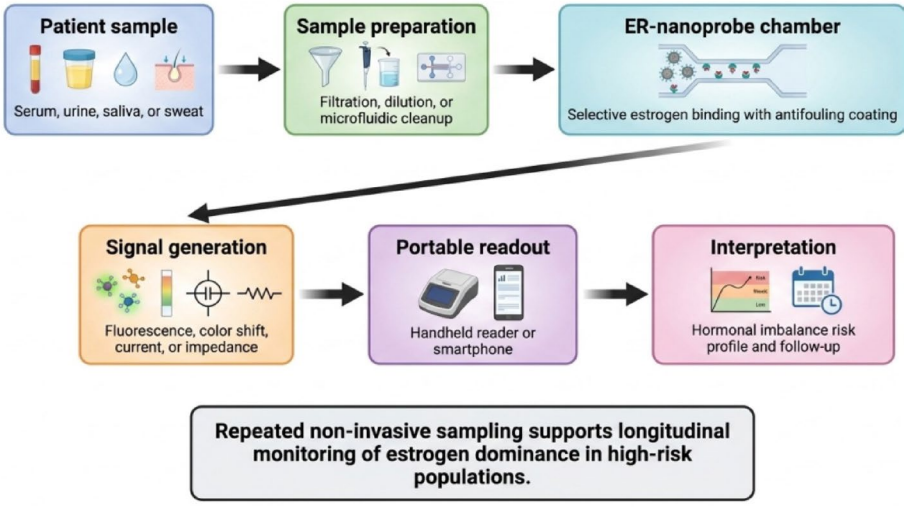


Fig. 6 Non-invasive biofluid detection workflow using ER-functionalized nanoprobes: Schematic workflow for the detection of estrogenic compounds in non-invasive biofluids like saliva, urine, sweat or serum using ERα/ERβ-functionalized nanoprobes. The workflow illustrates the importance of sample collection and interactions between estrogen and nanoprobwes, as well as signal transduction and data interpretation for future point-of-care monitoring of unopposed estrogen exposure and estrogen-related cancer risk

6.3 SPR (surface plasmon resonance), and SERS (surface-enhanced raman scattering)

SPR and SERS are the robust techniques of optical label-free detection that have real-time application. SPR is an optical technology that quantifies variations in the refractive index fluid at a dielectric-metal interface typically through a gold or silver-coated sensor chip [273–275]. In the presence of immobilized ER-functionalized nanoparticles on the surface, binding of estrogen molecules causes a change in local refractive index, and the condition of plasmon resonance changes [276]. This enables dynamic measurement of estrogen-ER complexes without requiring tags of fluorescence or enzymatic nature. The signals of Raman scattering are amplified by SERS through the plasmonic nanostructures like gold nanostar or silver nanorods. The binding of the hormone to ER-functionalized nanostructures revealed typical vibrational fingerprints of the estrogen which can be identified with ultra- high sensitivity [104]. SERS provides the single-molecule detection and outstanding multiplexing abilities due to thin spectral ranges utilization.[276]. Because of these properties can be applied in the detection of early stage cancer and low hormone levels imply subtle pathological changes. Figure 7 illustrates the principal signal-transduction mechanisms employed in estrogen receptor-functionalized nanoprobe biosensors. The schematic compares FRET-based fluorescence, electrochemical sensing, surface plasmon resonance, and surface-enhanced Raman scattering platforms. In each system, specific binding of 17β-estradiol to immobilized estrogen receptors produces a measurable optical, electrical, or plasmonic response. The figure highlights receptor recognition, nanomaterial-assisted signal amplification, and representative analytical readouts, demonstrating how molecular binding events can be translated into sensitive and selective estrogen detection for potential diagnostic applications and monitoring.

6.4 New age smart wearables and lab-on-a-chip devices

Innovations in the sphere of microfabrication and the wireless technology recently allowed the creation of the integrated biosensing platforms smart wearables and laboratory-on-a-chip (LOC) systems [277]. Such platforms integrate miniaturized signal

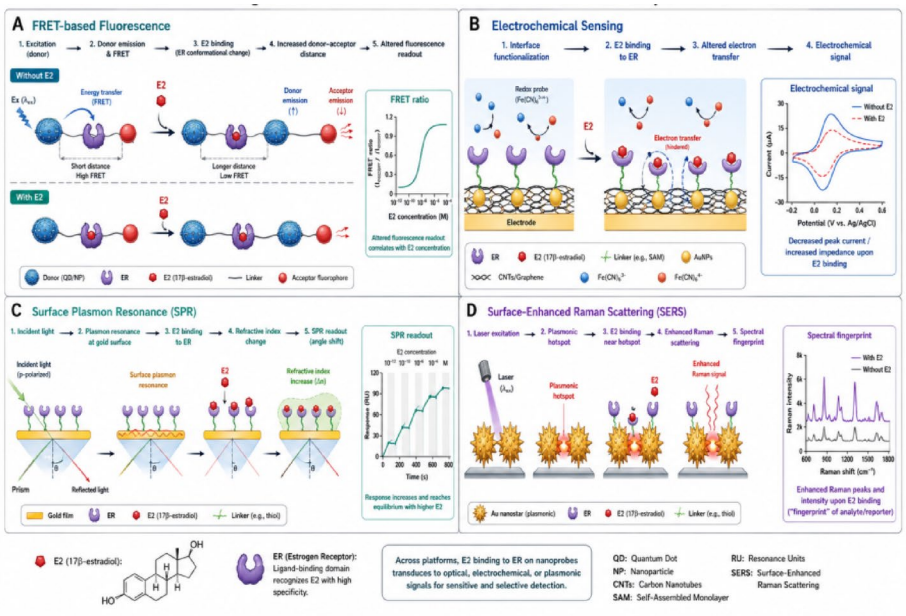


Fig. 7 Mechanistic signal transduction in ER-functionalized nanoprobe biosensors: Figure seven summarizes how ER-functionalized nanoprobes convert 17β-estradiol binding into fluorescence, electrochemical, SPR, or SERS signals. Signal direction depends on receptor conformation, surface chemistry, assay format, and nanomaterial design. E2, 17β-estradiol; ER, estrogen receptor; QD, quantum dot; AuNP, gold nanoparticle

transduction, wireless data readout with nanoprobe-based estrogen sensing. An example is microfluidic LOC devices have been developed that analyze estrogen concentrations in microliter-size biological samples such as sweat or saliva real-time using ER-functionalized nanoprobes [278]. Textile and skin patch wearable biosensors can constantly detect physiological biomarkers, and the data can be wirelessly transmitted to smartphones or cloud providers to provide clinical clinicians with remote access to analysis [279]. Typically, such systems have flexible electronics, electrochemical, or fluorescence identifications. These innovations facilitate decentralized screening in cancer and personalized health monitoring, mostly in the underserved or remote environments. Nanotechnology, wireless data analytics and microfluidics are converging to establish next-generation cancer diagnostics that are patient centric, portable and non-invasive [280].

For additional insight into the various modalities that could be used to explain how the estrogen receptor-functionalized nanoprobes transduce and report the signal of detection, a tabulated comparison (Table 4) of the main signal transduction and data readout mechanisms that are applied by different biosensing platforms are also described. Examples of these support mechanisms are optical (e.g. FRET, fluorescence, SPR, SERS), electrochemical (e.g. amperometry, voltammetry), mechanical (e.g. piezoelectric), and new hybrid systems (e.g. lab-on-a-chip, wearable sensors). All the approaches are described in terms of the signal type, the principle of detection, the materials used to functionalize, the level of sensitivity, as well as the contexts of application. As shown in Fig. 8, smart wearable and lab-on-a-chip systems can integrate ER-functionalized nanoprobes with microfluidic sampling, signal transduction, wireless readout, and AI-assisted interpretation for real-time estrogen monitoring.

Table 4 Comparative analysis of signal transduction and data readout modalities in estrogen receptor-functionalized nanoprobe biosensors

No	Detection method	Signal type	Key mechanism	Functionalization material	Sensitivity level	Application context	References
1	FRET-based Fluorescence	Optical	Donor–acceptor energy transfer	QDs, ER-protein conjugates	High	Multiplex biomarker imaging	[281, 282]
2	Quantum Dot Fluorescence	Optical	Light emission from nanocrystals	CdSe/ZnS QDs + ERs	Very High	Real-time hormone tracking	[283, 284]
3	Time-Resolved Luminescence	Optical	Lanthanide long-lived emission	Europium-labeled ERs	High	Tissue imaging, serum assays	[285]
4	Amperometric Electrochemical	Electrical Current	Redox reaction upon binding	AuNPs/ER-modified electrodes	High	Point-of-care diagnostics	[286, 287]
5	Voltammetry (DPV/CV)	Electrical Current	Potential-driven redox scanning	CNTs, graphene-ER hybrids	Moderate–High	Serum estrogen profiling	[268, 288]
6	Surface Plasmon Resonance (SPR)	Optical	Refractive index shift	Gold chip + ER coating	High	Label-free kinetic studies	[289, 290]
7	Surface-Enhanced Raman Scattering (SERS)	Optical (Raman)	Enhanced molecular vibration	Ag/Au nanostructure + ER	Ultra-high (single-molecule)	Early detection in biofluids	[291, 292]
8	Microfluidic LOC Devices	Electrochemical/ Optical	Integrated fluidics and detection	PDMS chips + ER-functionalized NPs	High	Portable cancer diagnostics	[293, 294]
9	Smart Wearable Sensors	Wireless/ Electrical	Continuous monitoring with feedback	Printed electrodes + ERs	Moderate–High	Remote hormone monitoring	[295, 296]
10	Multiplex Optical Biosensor Arrays	Optical	Multiple signal readouts	ER-tagged multi-QD chips	High	Parallel biomarker detection	[297, 298]
11	Fluorescent Microbead Assays	Optical	Dye-conjugated nanoparticle emission	Polymer beads + ERs	Moderate–High	Batch detection in microarrays	[299]
12	Electrochemiluminescence (ECL)	Optical/ Electrical	Light emission during redox cycling	ER-functionalized Ru-complexes	Very High	Ultra-sensitive hormone assays	[300]
13	Piezoelectric Biosensing	Mechanical	Mass-induced frequency shift	Quartz crystal + ER surface	Moderate	Real-time binding kinetics	[301, 302]
14	Optical Fiber Biosensors	Optical	Light signal modulation through fiber core	ER-modified tapered optical fibers	High	In vivo estrogen detection	[303, 304]
15	Photonic Crystal Biosensors	Optical	Resonance wavelength shift due to binding	Nanostructured ER-coated substrates	High	Multiplex screening platforms	[268, 305]

Sensitivity levels: Very High- indicates femtomolar or single-molecule range; High- is picomolar–nanomolar; Moderate- is nanomolar–micromolar

QDs quantum dots, *ER* estrogen receptor, *AuNPs* gold nanoparticles, *CNTs* carbon nanotubes, *PDMS* polydimethylsiloxane, *DPV/CV* differential pulse/cyclic voltammetry, *ECL* electrochemiluminescence, *SPR/SERS* surface plasmon resonance/ surface-enhanced raman scattering

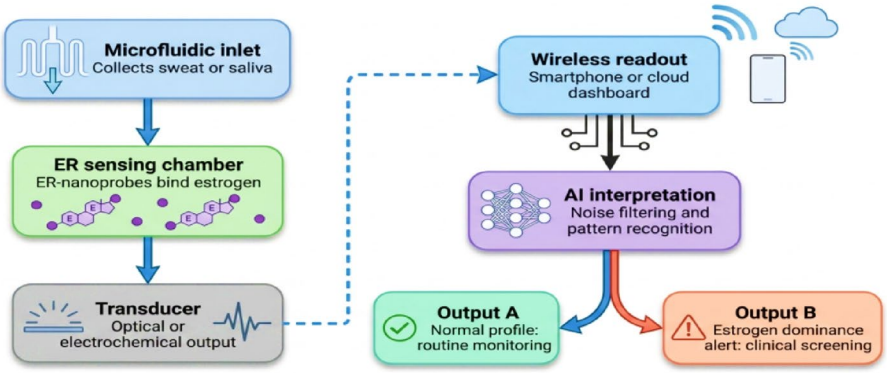


Fig. 8 Smart wearable and lab-on-a-chip integration for real-time estrogen monitoring: Integrated estrogen monitoring system concept, where sweat or saliva is funneled into the device via a microfluidic inlet, enters an ER-functionalized sensing chamber, and its concentration is quantified. The estrogen–receptor interactions are translated in an optical or electrochemical signal that is transmitted wirelessly to a smartphone or cloud-based dashboard and interpreted using AI-enabled noise-filtering and pattern recognition technology. Either the system can be used for routine hormone measures, or when estrogen(dominance) hormone patterns are detected, clinical screening alerts can be triggered

7 Clinical translation and diagnostic potential in estrogen receptor-functionalized nanoprobe biosensors

7.1 Diagnostic accuracy and validation

Nanoprobe biosensors functionalized with estrogen receptors (ER) are of high potential in increasing the accuracy of hormonal cancer diagnosis. At the epicentre of their clinical usefulness is high-level sensitivity, specificity, and predictive performance validation [306]. The term sensitivity concerns how accurately the biosensor can observe even the tiniest amounts of estrogen or biomarkers involving ER, and since it is essential when detecting a disease, it is paramount to be as sensitive as possible [307]. Specificity instead refers to the capacity of the biosensor to sort estrogenic compounds and other similar structure hormones or interfering biomolecules, minimizing the instance of false positive [22]. Receiver operation characteristics (ROC) curve based analysis designs a detailed receiver operating characteristic which in turn provides a measure by which the sensitivity/specificity compromise may be quantified and adopts a value that provides a record of the area under the curve (AUC) as a reference to measurement of the diagnostic reliability [308].

Compared to traditional methods which include enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry (IHC), ER-functionalised nanoprobes exhibit superior kinetics, resolutions and multiplexing ability [309]. Although ELISA is a well-established means to quantify estrogens, it lacks spatial resolution, and large volumes of samples are needed [173]. Immunohistochemistry is labour-intensive, semi-quantitative and despite being considered the gold-standard in receptor expression in tissue, is not in itself a quantitative analysis. It is possible to overcome these drawbacks through real-time, label-free, and highly quantitative analysis [309]. Nanoprobe biosensors can be used with microfluidic systems, which provides an advantage in the point-of-care application [310, 311]. To be accepted as a routine clinical diagnostic modality, their performance should meet, or even surpass those of accepted diagnostic modalities. Analytical performance in buffer cannot be assumed to be predictive of analytical performance in serum, urine, saliva or tissue-derived samples and calibration in the biological matrix of interest must be performed. Therefore, it is recommended that calibration curves/

matrices matched to the intended application, spike and recovery studies, dilution linearity studies, and matrix-effect analysis should be part of every ER-functionalized nanoprobe biosensor. Ideally recovery values should be reported for low, medium and high estrogen levels. Correlation analysis, Bland–Altman) plots and clinically relevant decision limits should be used to assess agreement with established reference methods, particularly LC–MS/MS or validated immunoassays.

7.2 Application in screening high-risk populations

The increasing incidence of cancers which are caused by estrogen forces the development of better screening methods, especially on high-risk populations. Along with such aspects as polycystic ovary syndrome (PCOS), obese, and postmenopausal females populations that display delayed exposure to estrogen or estrogen excess and ER-functionalized nanoprobe biosensor offers a paradigm shift in early detection test [312, 313]. In particular, a decrease in the progesterone levels is noticed in postmenopausal women, which results in unopposed estrogenic activity and predisposes to endometrial and breast cancers [314, 315]. On the same note, obesity leads to peripheral conversion of androgens to estrogens through aromatase in the adipose tissue whereas PCOS patients tend to have anovulatory cycles and chronic exposure to estrogens [316, 317].

Nanoprobe-based bio-sensors that would allow direct estimation of estrogen level or ER activity in blood, saliva, urine, or other bodily fluid would offer a non-invasive real-time measure of hormonal imbalance [205]. This potential will allow the regular screening, risk stratification and prompt referral to confirmatory diagnostics or preventive treatment. Moreover, the biosensors could be used as companion diagnosis in hormone replacement therapy (HRTs) and selective estrogen receptor modulators (SERM) [318]. Endocrinologist and oncologists can calculate individual doses and evaluate the therapeutic benefit or hazard by checking the amount of circulating estrogen or the involvement of estrogen receptors, hence the shift to precision medicine in endocrinology and cancer. They could be integrated into a wearable or a portable diagnostics platform that would allow them to be used in a decentralized manner at home, eliminating healthcare burden and allowing longitudinal monitoring [205]. If analytically validated and clinically confirmed in prospective high-risk cohorts, ER-functionalized nanoprobe biosensors could support earlier risk stratification and referral for confirmatory diagnostics. However, their use in population or high-risk screening should currently be considered a future translational goal rather than an established clinical application. Prospective studies are needed to define clinically meaningful cut offs, diagnostic sensitivity and specificity, ROC-AUC, predictive values, reproducibility across sites, and whether biosensor-guided monitoring improves patient outcomes.

Though biosensors with ER functionalized nanoprobe are promising, they have not been widely tested with patient specimens or prospective clinical populations. There is not enough research available in human subjects other than buffer systems, spiked biofluids, cell-model systems, or small preclinical research cohorts. So it can be considered a future translational application, rather than a clinical practice, to use their proposed use for screening women with obesity, polycystic ovary syndrome, menopause or longer durations of estrogen exposure. Additional multicenter studies are needed to establish clinically useful cut offs, correlate with LC–MS/MS or validated immunoassay, and

ascertain ability to enhance diagnosis, risk stratification, and patient outcomes when using biosensors for monitoring.

7.3 Regulatory and standardization challenges

Nevertheless, ER-functionalized nanoprobe biosensors have great potential but there are several regulatory and standardization challenges on the road to transitioning them to the bedside. Agencies like the U.S. Food and Drug Administration, (FDA), the European medicines Agency (EMA), demand a large set of evidence of safety safety, efficacy and reproducibility before approves any diagnostic device to be used in the clinic [22]. Biosensors particularly nanotechnology based biosensors are further under confrontation because of their possible issues of biocompatibility and long term management together with non-characterised toxicological profiles of nanomaterials [319].

Another significant issue here is to achieve batch-to-batch reproducibility of the functionalized nanoprobes since these operations involve many sources of variability in the surface modification, bioconjugation steps [320]. Uniform methods to create nanoparticles, functionalize them with ER ligands or antibodies, and use as a signal readout system, ought to be designed and verified. To address the requirement of Good Manufacturing Practice (GMP) and ISO, quality control measures such as calibration standards, shelf-life test, and inter-laboratory reproducibility test are required [320]. Biosensor clinical trial design also varies as compared with pharmaceutical trials. Rather than dose–response studies, there is the focus on diagnostic performance in a variety of patient populations [321]. Multi-phase clinical studies should show that biosensors would be able to effectively detect biomarkers both within physiological and pathological limits, in different environmental temperatures, pH, interferences. Moreover, the connections to the digital health platforms necessitate adherence to security requirements of data confidentiality, network safety, and interoperability [322, 323].

There is a roll out in harmonizing biosensor regulatory frameworks all around the world, some of which is the FDA nanotechnology regulatory science research plan [324]. The key will be to engage early with the regulators, implement modular approaches to validation, participate in consensus guidance document development with professional societies and standardization organizations (e.g., ASTM, ISO), etc. Solving these regulatory and standardization problems is the initial step toward broad clinical distribution of ER-functionalized nanoprobes and sustainable embedding into the diagnostic routine.

8 Challenges and future directions in estrogen receptor-functionalized nanoprobe biosensors

8.1 Technical barriers

With regard to estrogen receptor (ER)-functionalized nanoprobe biosensors, maintaining stability in physiological conditions, including changes in pH, temperature, and ionic strength can be listed in the top technical barriers. Nanoprobes can aggregate or get damaged that decreases the sensing efficiency. There is also the problem of reproducibility; surface chemistry and conjugation methods provide inconsistencies between the batch to batch reproducibility of the ER surfaces. This variability weakens reliability and standardization in the clinical field. Moreover, signal drift and biofouling-mediated loss of sensitivity may be able to influence the diagnostic performance. The solution to such problems will involve well-developed material engineering, strong surface coatings,

and common procedures in nanoprobe manufacturing and veracity. Reproducibility should be checked on several levels from the synthesis of nanoparticles to immobilization of receptors, surface blocking, transduction of the signal to the interpretation of the results. Also checked if there is batch-to-batch variation for the nanoparticle size, surface charge, receptor density and ligand chemistry, which can influence sensitivity and selectivity significantly. Hence, intra-assay and inter-assay coefficient of variation, electrode-to-electrode or chip-to-chip variability, inter-operator and inter-laboratory (where possible) should be reported in future studies.

8.2 Biological and clinical limitations

Biological complexity is an issue that can constrain the clinical performance of ER-functionalized nanoprobe biosensors. Binding specificity may be disrupted by endogenous hormones and metabolites, such as phytoestrogens, cortisol, thyroid hormones, or a false positive or false negative may be produced. Heterogeneity of tumors introduces additional issues, because cancer cells can have different quantities of estrogen receptors or change the phenotype in the course of time. Furthermore, reading of the hormone levels in the body is complex and fluctuates depending on the circadian rhythms, drugs, and illness condition. These biological parameters will require highly specific, rather flexible, and real-time adjustable biosensors in different heterogeneous patients.

8.3 Future innovations

Promising new innovations, new limitations of the ER-functionalized nanoprobe biosensors can be overcome. Machine learning and artificial intelligence (AI) may be utilized to interpret signals, suppress noise, and detect barely noticeable patterns in bio sensor measurements. Diagnosis and stratification can be enhanced by identifying ER with dual-targeting biosensors that specifically identify other markers such as progesterone receptor (PR) or HER2. Also, combinations of biosensors with imaging modalities and therapeutic platforms e.g., in theranostic nanodevices, allow simultaneous diagnosis and therapy. These innovations are moving the field towards customized and non-invasive and real time detection and management of cancer an innovative leap in diagnostics of hormonal cancer.

AI-assisted interpretation, multiplex hormone profiling, imaging integration, and theranostic ER-functionalized nanoprobes are promising, but technical and logistic factors are still obstacles to clinical translation. In order to prevent overfitting and biased interpretation, AI models need large datasets with well-defined annotations, multi-center, and external validation. Multiplex sensors can have interfering signal cross-talk, varying calibration parameters for each analyte, and may have challenges separating free estrogen, bound estrogen and metabolized estrogen. For imaging or theranostic nanoprobes, there are further challenges in dose optimization, biodistribution, clearance, repeated-exposure safety, as well as nanomaterial toxicity and immunogenicity, and the question of whether the levels of signal intensity are more related to estrogen concentration, expression of the ER, uptake of a probe, or perfusion of tissue. The limitations should be solved by antifouling surface engineering, by ratiometric or dual-signal readout, by using common reference materials, by performing matrix-matched calibration, by using orthogonal confirmation by LC–MS/MS, and by performing validated immunoassays

and stepwise validation in buffer, biofluids, ex vivo tissues, animal models, and prospective clinical cohorts.

The distinction between the near, medium, and long-term forms of application needs to be identified in a realistic translation roadmap. Laboratory-based or microfluidic ER-nanoprobe assays, with reference hormone assays are more likely to show achievable results in the laboratory. Medium-term development could involve portable point-of-care platforms, that have established clinical cut-off values, calibrated, and reproducible for high-risk groups. However, more long-term applications, including wearable continuous monitoring, AI-guided interpretation, imaging-integrated ER probes and theranostic nano-devices, are more speculative and will need large volume safety, pharmacokinetic, regulatory, and clinical outcomes testing before entering into human use. But these platforms should be communicated as "milestones in translation", not as imminent clinical solutions, given that extensive analytical validation, safety testing, standardized manufacturing, regulatory clearance and prospective clinical data will be needed before they become clinically available.

9 Conclusion

Unopposed estrogen is the key to the pathogenesis of the hormone-related malignancies that includes endometrial, ER-positive breast, and some ovarian cancers. It is critical that the existing diagnostic prompts fall short in the ability to conduct subtle, tissue-specific diagnosis of hormonal imbalance due to low sensitivity. The nanoprobes (ER-nanoprobes) function as a revolutionary means to solve this problem because they provide the molecular specificity of estrogen receptors together with the response and sensitivity of nanomaterials through signal amplification. All these high-sensitive and non-invasive advanced biosensors can make possible in real-time monitoring of free estrogens in biological systems, which is the key to future early cancer detection and intervention.

This review has provided the narrative view over the most recent innovations in the field of ER-nanoprobe technologies considering their design principles, approaches to receptor functionalization, taxonomies of used nanomaterials and a wide array of signal transduction mechanisms, starting with fluorescence down to electrochemical and plasmonic approaches. They can be used in different contexts, such as laboratory based assays and cellular imaging and biofluid analysis or as wearable platforms and thus have great potential to adapt to both clinical and research use. ER-nanoprobes have great potential, despite the present-day technical and biological problems, such as stability in physiological environment, cross-reactivity of similar biomolecules, heterogeneity of tumors.

Possible future developments are the introduction of signal processing through artificial intelligence, multiplex biosensors to profile a wider range of hormones and to use theranostics by merging the detection with the selective therapy. ER-functionalized biosensing has the potential to usher in a whole new era of precision medicine, overcoming the limitations of current practice by supporting an earlier diagnosis, personalization of monitoring, and perhaps best of all, an improvement in outcome for patients at risk of estrogen-driven cancers, as a result of the triple convergence of nanotechnology, endocrinology and oncology. Overall, ER-functionalized nanobiosensing represents a promising but still emerging approach for estrogen-related cancer risk assessment. Its clinical value will depend on rigorous analytical validation, reproducibility across laboratories,

safety and stability testing, regulatory clearance, and prospective studies demonstrating that biosensor-guided decisions improve diagnostic accuracy or patient outcomes.

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