Are aptamer-based biosensing approaches a good choice for female fertility monitoring? A comprehensive review

Are aptamer-based biosensing approaches a good choice for female fertility monitoring? A comprehensive review.

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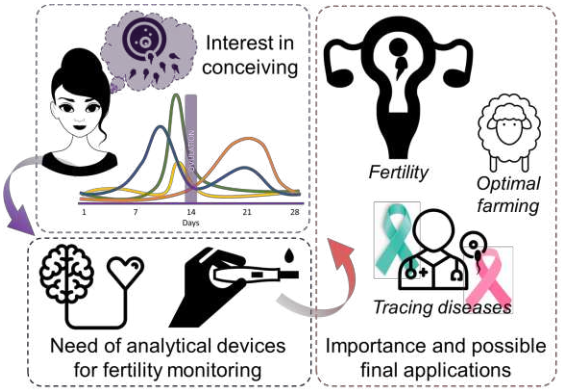
Abstract

The WHO estimates that 8-10% of couples are facing fertility problems, often due to inaccuracy in predicting the female's ovulation period controlled by four key hormones. The quantification and monitoring of such key hormones are crucial for the early identification of infertility, but also in improving therapeutic management associated with hormonal imbalance. In this review, we extensively summarize and discuss: ***i)*** drawbacks of laboratory methods for fertility testing (costly, invasive, complex) and commercially available point-of-care tests (measuring only one/two of the four key hormones), ***ii)*** the understanding of different biosensors for fertility monitoring, and ***iii)*** an in-depth classification and overview of aptamer-based sensing of the hormones of interest. This review provides insights on hormone detection strategies for fertility, with a focus on the classification of the current 'aptasensing' strategies, aiming to assist as a basic guide for the development of accurate fertility window monitoring tools based on aptamers.

6 Keywords

Fertility monitoring, Biosensors, Aptamers, Estradiol, Progesterone, Gonadotropin-releasing hormones.



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1. Introduction

According to the World Health Organization (WHO), approximately 8-10% of couples face fertility problems, meaning more than 50 million people worldwide are struggling to conceive (Alawan et al., 2020). This has a tremendous impact on society and couples' lives, both financially (healthcare, personal budget, etc.) and psychologically. The fertility of men can be tested by simple semen analysis and remains active for nearly all their adult life. In contrast, female fertility is periodic and completely disappears in later life (Zia et al., 2013). The reproductive endocrine system controls hormone levels regulating functions associated with sex, development, and the reproductive system. Female reproductive endocrine disorders are caused by alterations in the hypothalamus-pituitary-gonadal axis and can result in various symptoms and medical conditions, including infertility, amenorrhea, hirsutism, uterine fibrosis, cancers, and virilization, among others (Huang et al., 2021; Leva et al., 2002; Liang et al., 2019; Sun et al., 2021).

Therefore, the accurate quantification and monitoring of specific female hormones are crucial, not only for the early identification of infertility but also for improving the tracing of diseases associated with a hormonal imbalance (e.g., ovarian or breast cancer) (Khelifa et al., 2022; Leva et al., 2002). A primary reason couples have difficulty conceiving is their inability to accurately predict the female's ovulation period. Ovulation is a response to different hormone concentrations in the blood and occurs periodically. However, fertility hormone levels constantly change (**Fig. 1a**) (Khanwalker et al., 2019). These fluctuations in reproductive hormone levels can be caused by numerous factors, including menstrual cycle stage, age, sex, and pregnancy (Chang and Yeh, 2021).

Infertility in women can be considered a multifactorial mechanism associated with different factors and disorders (which might influence hormone levels) such as age, cervical, tubal or peritoneal factors (Alawan et al., 2020; Khanwalker et al., 2019), polycystic ovarian disease (Deswal et al., 2019; Torre et al., 2014) or endometriosis (De Ziegler et al., 2010; Zullo et al., 2017). Endometriosis is an estrogen-dependent chronic disease that affects 10-15% of women aged 25-35 years old (Macer and Taylor, 2012). Significantly, around 25-50% of infertile women have endometriosis and 30-50% of women with endometriosis are infertile (Bulletti et al., 2010).

Today's commercially available fertility tests consist of lateral flow bioassays (Khelifa et al., 2022) that use the principle of immunoreactions with optical readout (based on nanoparticle-linked antibodies) through a disposable dual test stick (such as Clearblue® and Mira®), only detecting luteinizing hormones (LH) and/or estrogen metabolites. However, key hormones related to women's fertility include: ***i)*** follicle-stimulating hormone (FSH), ***ii)*** estradiol (E2)

which is the main form of estrogen, ***iii)*** progesterone (P4), and ***iv)*** LH. Hence, three major hormones are currently not actively detected, even though their role is indispensable for ovulation and fertility. Therefore, highly specific detection and quantification of these four key hormones can produce an accurate timeline of a woman's menstrual cycle, identifying the optimum time to conceive in a personalized manner (Khanwalker et al., 2019). Moreover, knowing how these reproductive hormones fluctuate throughout the ovulation cycle provides relevant information and potential usefulness in gynecologic oncology. Remarkably, the fulfillment of hormone monitoring would make it possible to address the third sustainable development goal (SDG3) of the United Nations, particularly targeting 3.4 and 3.7 in promoting well-being and ensuring universal access to sexual and reproductive healthcare services by 2030.

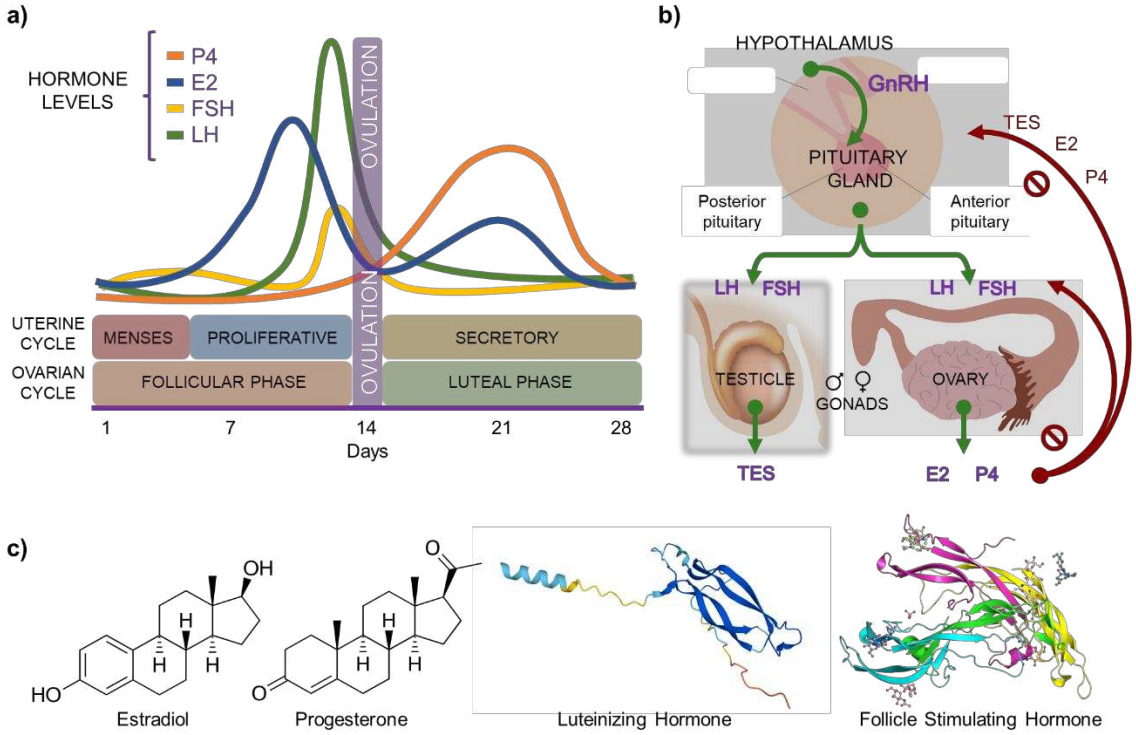


Fig. 1. Schematic representation of **a)** the fluctuation of key fertility hormones with the uterine and ovarian cycles and **b)** regulation of reproductive function by GnRH. GnRH is first released from the hypothalamus and interacts with specific receptors on the plasma membrane of the pituitary gonadotropin. This promotes the secretion of gonadotropins LH and FSH. The gonadotropins regulate masculine and feminine gonad (testicle and ovary) functions, producing testosterone (TES), E2, and P4. The GnRH-like compounds may interact directly with the gonads to inhibit steroidogenesis. **c)** Chemical structures of the four key hormones (obtained from uniprot.org)

The 'fertility window' of women is a period of the ovulatory menstrual cycle in which the woman owns the potential to conceive (Zia and Mukhopadhyay, 2016). The hormonal cycle (**Fig. 1a**) begins as a response to low levels of estrogen when the hypothalamus sends a signal (i.e.,

gonadotropin-releasing hormone (GnRH)) to the anterior pituitary gland to secrete FSH (**Fig. 1b**) at the end of the follicular phase (Serafín et al., 2019). As FSH levels rise, a dominant follicle will emerge. However, FSH levels decline before the egg bursts, requiring LH (secreted by the pituitary gland) for the final step prompting the follicle to rupture and trigger ovulation. The secretion of P4 by the granulosa cells (in the ovary) occurs in the later stages of ovulation (luteal phase) and is responsible for the thickening and maintenance of the endometrium (uterine wall). If fertilization does not occur, the *corpus luteum* disintegrates and P4 production ceases, causing endometrial tissue to disperse as menstruation (beginning of follicular phase). E2 also stimulates endometrial thickening and it decreases when the *corpus luteum* disintegrates (Khanwalker et al., 2019) triggering a new cycle (Serafín et al., 2019). **Table 1** summarizes the female healthy levels of the four hormones in different stages of the cycle and life of women. Overall, the interdependent four hormones are essential to accurately monitor the fertility window.

Table 1. Female healthy blood plasma levels of the corresponding fertility-related hormones in different stages of life and phases (Ph.) of the ovarian cycle.

Hormone	Prepubescence	Follicular Ph.	Ovulation	Luteal Ph.	Menopause	Pregnancy
E2	< 70 pM	55 pM – 1.9 nM	110 – 550 pM	70 – 590 pM	0 – 110 pM	690 pM – 9.2 nM
P4	0.3 – 1 nM	0.3 – 2.2 nM	Up to 64 nM	6.4 – 79.5 nM	---	32 – >954 nM
LH	---	1.9 – 14.6 IU/L	12.2 – 118 IU/L	0.7 – 12.9 IU/L	5.3 – 65.4 IU/L	< 1.5 IU/L
FSH	0 – 4 IU/L	1.4 – 9.9 IU/L	6.2 – 26 IU/L	1.1 – 9.2 IU/L	19 – >100 IU/L	< 1.7 IU/L

*Note: levels obtained from (Alhadrami et al., 2017; Huang et al., 2021; Khelifa et al., 2022; Mayo Clinic Laboratories, 2022; University of Rochester Medical Center Rochester, 2022a, 2022b; Zinaman, 2020)

2. Pressing need for easy-to-use and accurate analytical tools

Apart from their crucial roles in fertility regulation, most reproductive hormones are considered endocrine-disruptive chemicals (EDCs) so many research groups have invested time and resources to develop efficient detection and quantification methods for tracing hormones in different environmental and food samples. Environmental EDCs are released from various municipal, industrial, institutional and agricultural sources via wastewater, solid waste, and animal waste streams (Akki et al., 2015). Given their impact on our health, there is a pressing need and ever-growing interest in developing easy-to-use and accurate analytical tools to assess EDC levels in different types of samples (Prante et al., 2020). Other than environmental monitoring, the measurement of certain hormones is crucial to obtain chemical information about the human/animal body including the ability to conceive (animal reproduction and optimal farming). Importantly, reproductive hormones are the second most commonly measured hormones after thyroid hormones in clinical laboratories (Wheeler, 2013).

Conventionally, the clinical detection and quantification of all types of hormones (including the reproductive hormones discussed in this review) have been reliant on antibody-based techniques for many decades. However, these methods include suboptimal inter-assay reliability and reproducibility, high reagent costs, high sample volumes required (limiting the number of samples taken from a single subject), and the time-consuming nature of antibody-based assays (Weller, 2016). Additionally, it is important to remember that the production of the required antibodies needs an animal host, which presents difficulties as the molecule of interest may be toxic to the organism or have little inherent immunogenic response (Jayasena, 1999; McKeague and DeRosa, 2012). To overcome these issues, alternative methods of hormonal detection and quantification have been developed such as the replacement of the antibodies with synthetic homologs called aptamers (summarized in **Section 3**) (Izzi-Engbeaya et al., 2020). In contrast with the aforementioned drawbacks, aptamers are advantageously more stable than antibodies due to the rigidity of the phosphodiester backbone. Aptamers are produced chemically with high reproducibility and purity, which reduces production costs and allows for introducing modifications. For example, aptamers are < USD 50/g compared with ~USD 300/g for recombinant monoclonal antibodies produced using mammalian cell lines (Hammerschmidt et al., 2014; Izzi-Engbeaya et al., 2020; Walter et al., 2012). Besides, high temperatures, harsh pH conditions, or drastically high levels of salt would irreversibly denature antibody proteins, while an RNA or DNA aptamer would merely unfold temporarily (see **Section 3**) (Cánovas et al., 2022; Chen and Yang, 2015).

2.1. Conventional methods of fertility monitoring

Current methods of fertility monitoring include physical or vaginal exams, laparoscopy, ultrasound scans of the ovary, or evaluation of hormone levels by blood analysis (Zia and Mukhopadhyay, 2016). These techniques are broadly used to identify whether a woman is capable of conceiving, however, all these methods are characteristically invasive, which can cause pain and discomfort to the patient.

Although the standard analyses are traditionally performed in blood plasma, recently, these test methodologies offer possibilities for analyses in other biological fluids obtainable less invasively (urine, sweat, saliva, tears, and skin interstitial fluid) (Yáñez-Sedeño et al., 2020). Since the concentration of hormones in biological fluids is in the pico–nanomolar range (pM–nM) (Yáñez-Sedeño et al., 2020), several methods have been applied to detect hormones with high sensitivity and specificity (Bahadir and Sezgintürk, 2015): high-performance liquid chromatography (HPLC) using various detectors including UV (Wang et al., 2006), fluorescence (Trapiella-Alfonso et al., 2011) and electrochemical (EC) (Arvand and Hemmati,

2017), HPLC coupled with mass spectrometry (HPLC–MS), gas chromatography-mass spectrometry (GC–MS), enzyme-linked immunosorbent assay (ELISA) tests (Wang et al., 2007), and radioimmunoassay (RIA) (Valentini et al., 2002). For example, urinary E2 levels are frequently detected by biological assays, including ELISA and RIA (Triviño et al., 2019). The analysis of the rate of excretion of hormonal metabolites, e.g., estrone glucuronide (E1G) and pregnanediol glucuronide (PG), in urine is non-invasive and has proven to be highly correlated to the hormonal concentration in the ovary (Zia and Mukhopadhyay, 2016). However, fluctuations in the metabolite concentrations are common in urine, and therefore 24 h collection of the sample is recommended.

RIA is the most common method among the existing laboratory-based techniques. Albeit being one of the most accurate, this assay has major drawbacks including the production of radioactive waste, high cost, time-consuming, and requiring trained professionals (Zia and Mukhopadhyay, 2016). On the other hand, there are several ELISA kits based on colorimetric sandwich-type immunoassays using fertility-related antibodies labeled with horseradish peroxidase (HRP-dAb) (Arévalo et al., 2021a, 2021b; Serafín et al., 2019). Despite avoiding radioactive waste, they do not allow HRP labels to approach the electrode surface to obtain efficient electron transfer between the HRP moiety and the electrode surface (Kokkinos et al., 2016). Moreover, ELISA kits do not typically produce quantitative results or precise simultaneous measurements of all four necessary hormones. Overall, existing methods and kits exhibit high complexity and a long analysis time. However, innovative point-of-care (POC) biosensing approaches have emerged in recent years to overcome such complexities and drawbacks of current methods (Romeo et al., 2016; Wang et al., 2016; Zarei, 2017). Such biosensor types could act, in the future, as rapid, at-home fertility monitoring tools alleviating apprehensiveness associated with routine screenings and giving women the privacy desired when trying to conceive.

Following the IUPAC convention, “a biosensor is a self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is in direct spatial contact with a transducer element” (Scheller et al., 2001). In general, when constructing biosensors, it is possible to classify them according to the transduction method used: ***i)*** **Electrochemical,** in which a chemical reaction between the recognition element and target analyte will produce a redox event that promotes changes of current, potential, impedance (Ebrahimi et al., 2022) or capacitance (Bahadir and Sezgintürk, 2015), ***ii)*** **Optical,** in which the recognition binding events are converted into optical measurable changes (e.g., color, fluorescence, refractive index or diffraction), ***iii)*** **Piezoelectric,** the transducers are based on changes of the molecular weight upon target binding, and ***iv)*** **Calorimetric** in which the changes in heat during an

exothermic reaction catalyzed by an enzyme can be monitored by calorimetric transducers (Prante et al., 2020). In this review, we focus on the first two options (electrochemical and optical biosensors) as they allow for low-cost POC tests and more specifically (**Section 3**) based on the use of aptamers as recognition elements.

2.2. Electrochemical monitoring of fertility

When compared to conventional analytical methods, electrochemical techniques have many advantages: low cost, high sensitivity, fast response, portable and easy operation. Thanks to the integration of bio-recognition elements, electrochemical biosensors are ideally suited for the specific and highly sensitive detection of hormones.

Mainly two different approaches (**Fig. 2**) have been pursued toward electrochemical hormone monitoring using: ***i)*** modified electrodes(Nodehi et al., 2020) based on graphene (Janegitz et al., 2014) or other nanocomposites (Govindasamy et al., 2019) where the reproductive hormones are directly oxidized (Pavinatto et al., 2018) (**Fig. 2a**) and ***ii)*** bio-recognition elements such as aptamers (Liang et al., 2019; Samie and Arvand, 2020), molecular imprinted polymers (Lahcen et al., 2017) or antibodies (Liu et al., 2012) for the specific recognition of the hormone of interest (**Fig. 2b**).

During recent decades, diverse antibody-based electrochemical biosensors for the single, or in some cases dual, detection of key fertility hormones have been reported (Arévalo et al., 2021b; Arvand and Hemmati, 2017; Serafín et al., 2019). In the last year, Arévalo *et al.* designed a complete array for the simultaneous electrochemical detection of four fertility-related hormones. The selected hormonal panel contained P4, LH, E2, and prolactin (PRL). The antibody-based platform involves direct competitive assays (P4 and E2) and sandwich assays (LH and PRL) on functionalized magnetic microbeads in combination with commercially available screen-printed carbon electrodes (SPCEs) (from DropSens). Amperometric transduction was obtained using an enzymatic H_2O_2/hydroquinone system (Arévalo et al., 2021a).

Further to enzymatic systems, numerous electrochemical transduction methods based on gold nanoparticle (AuNP)-coupled aptasensors have been reported (Du et al., 2016a, 2016b; Ni et al., 2017; Qiao et al., 2021) (more details in **Sections 3.1–3.2**) since the large surface area and high redox activity of AuNPs leads to signal enhancement and increased biosensor sensitivity (Prante et al., 2020)**.**

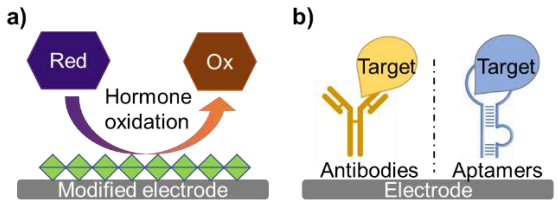


Fig. 2. Basic approaches pursued during electrochemical-based monitoring of hormones: **a)** direct oxidation of the hormone on the previously modified working electrode surface and **b)** indirect detection of the hormone of interest using specific bio-recognition elements such as antibodies or aptamers.

Impedimetric-based approaches have also been widely employed given the capabilities of electrochemical impedimetric spectroscopy (EIS) as an efficient non-destructive tool for the electrochemical investigation of bio-macromolecules. (Ebrahimi et al., 2022) However, despite substantial research efforts giving rise to significant achievements in detecting hormones and/or their metabolites, there are currently no examples of commercially available electrochemical biosensors for POC detection of reproductive hormones (Yáñez-Sedeño et al., 2020).

2.3. Optical monitoring of fertility

Optical approaches offer simplicity in terms of readout. The biomolecular interaction on the sensor surface modulates the light characteristics of the transducer (i.e., intensity, phase, polarization, etc.), and the biosensing event can be detected by changes in diverse optical properties such as absorption, fluorescence, luminescence, or refractive index, among others. In addition, optical transducers have the potential for parallel detection, making array or imaging detection possible (Lechuga, 2005). To achieve all this, nanomaterials are key components in the sensing designs because they take on different roles acting as: ***i)*** optical elements, ***ii)*** nanostructured surfaces as carriers, and ***iii)*** facilitate catalytic activity useful for both signal generation and amplification (Kim et al., 2016). For example, aptamer-based sensing approaches in which AuNPs or quantum dots (QDs) are incorporated have been reported in the literature with promising sensitivity (Urmann et al., 2017).

AuNP-based biosensors often operate with an optical transduction method since the aggregation of the nanoparticles upon aptamer-target or antibody-target binding leads to a color change with a wavelength shift from 520 nm to 650 nm (Prante et al., 2020). The band at 520 nm corresponds to dispersed nanoparticles while the band at 620 nm corresponds to aggregated particles (Bottari et al., 2020). Initially, the single-stranded DNA (ssDNA) is adsorbed on the surface of the AuNPs to avoid nanoparticle aggregation (**Fig. 3**) (Carnerero

et al., 2017). Next, binding of the aptamer to its target induces aptamer release, and aggregation of AuNPs will occur upon the subsequent addition of salt (NaCl) (Alhadrami et al., 2017). Thus, the absorbance intensity will increase at 620 nm (aggregated AuNPs), while it decreases at 520 nm (dispersed AuNPs). It is important to take into account that: ***i)*** real samples usually contain salt and liposomes which limit the sensitivity of the colorimetric assay (Alhadrami et al., 2017), ***ii)*** in this type of assay the aptamer only interacts with the AuNPs via electrostatic interactions (so considered a label- and immobilization-free assay), and ***iii)*** certain targets can interact (adsorption) directly with AuNPs causing aggregation and the corresponding color change, demonstrating the inefficacy of some aptamers when the colorimetric approach is used (Bottari et al., 2020; Hou et al., 2021; Niu et al., 2022).

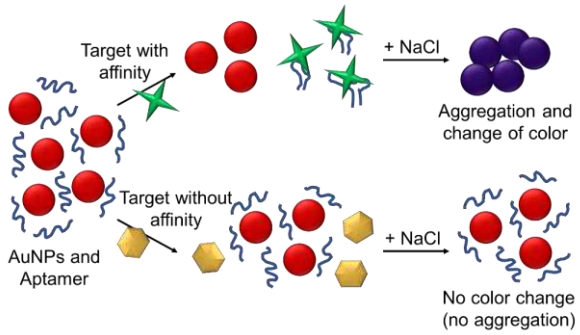


Fig. 3. Schematic representation of the colorimetric AuNPs assay using aptamers as recognition elements.

3. Aptamer-based (bio)sensing approaches

To overcome issues related to the commonly used antibody-based strategies, the employment of synthetic target-binding oligonucleotides (aptamers) has gained importance. In line with advancements in this technology, the development of aptamer-based assays has been accelerated over the last two decades (Izzi-Engbeaya et al., 2020). However, it is important to remark that careful characterization of the aptamer of interest is needed before its employment as a recognition element in a biosensor (Bottari et al., 2020; Zhao et al., 2021).

Aptamers are short ssDNA or RNA oligonucleotides first described in 1990, consisting typically of 40-100 nucleotides, and proposed as a promising alternative to commercially available antibodies (Cánovas et al., 2022). At optimized conditions, aptamers form a unique three-dimensional structure due to intramolecular interactions such as hydrogen bonding, electrostatic interactions, hydrophobic interactions, and Van der Waals forces between the nucleotides. This unique three-dimensional structure of the aptamer forms a binding site that enables the recognition of the target molecule. It is, however, important to remark that not all nucleotides of an aptamer are involved in forming the binding site (Cánovas et al., 2022; Yang

et al., 2017). Nevertheless, the non-binding domain of the aptamer may affect the binding affinity of the aptamer-target complex (Alhadrami et al., 2017; Cánovas et al., 2022).

Aptamers often undergo significant conformational changes upon aptamer-target binding, offering great flexibility in the design of novel biosensors such changes can be transformed into chemical, fluorescent, or color changes coupled with electrochemical, mass-sensitive, or optical methodologies (**Fig. 4a**) (Izzi-Engbeaya et al., 2020). Moreover, aptamers can be easily modified with diverse types of reporter molecules or dyes, enabling their integration into different biosensing and therapeutic platforms (Cho et al., 2009). Furthermore, aptamers can be selected against toxic targets or under non-physiological conditions as well as against molecules that do not elicit an immune response. Hence, the versatility offered by the selection of aptamers allows for tailored designs depending on the interest and the final application (Svobodová et al., 2017).

Aptamers have been developed to detect a wide variety of targets *in vitro* and *in vivo* (Izzi-Engbeaya et al., 2020) in a diversity of samples (**Fig. 4b**) for biosensing and therapeutic applications (Alhadrami et al., 2017). However, despite this broad exploration and use, only a handful of aptamers are commercially used (and approved) in diagnostics and therapeutics (Prante et al., 2020). For instance, the most prominent therapeutic aptamer is Macugen by Pfizer, which was approved by the FDA in 2004, for treating neovascular age-related macular degeneration (AMD) (Center for Drug Evaluation and Research, 2004). NeoVentures Biotechnologies Inc. commercialized an aptasensor to detect ochratoxin A and aflatoxins in food samples for the market (Tan et al., 2021). Other examples, used for anti-angiogenic purposes, are the aptamers for AMD treatment (Zimura, Fovista, and Pegnivacogin) which are in advanced stages of clinical trials, (Haßel and Mayer, 2019; Sun and Zu, 2015) along with a few more aptamers for other renal diseases (Kaur et al., 2018; Prante et al., 2020).

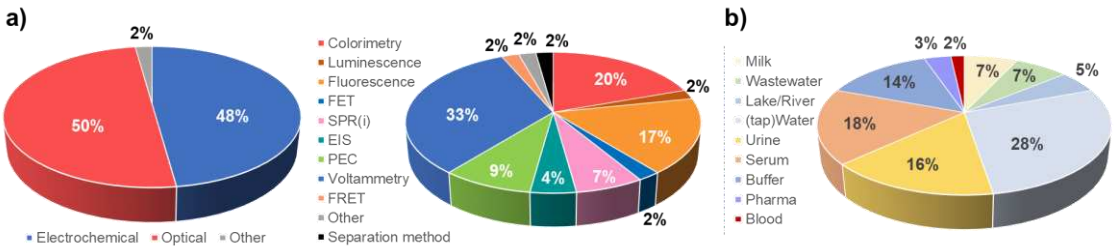


Fig. 4. Schematic representation of **a)** variability of analytical techniques (mostly divided into optical, and electrochemical) used in fertility-related hormone aptasensing approaches and **b)** different matrices tested in the literature. FET: field-effect transistors, SPR(i): surface plasmon resonance imaging, EIS: electrochemical impedance spectroscopy, PEC: photoelectrochemistry, FRET: fluorescence resonance energy transfer microscopy.

This review summarizes and discusses all aptamer-based sensing strategies for the monitoring of the four key fertility-related hormones (E2, P4, LH, and FSH) published up to date. Remarkably, all approaches are based on electrochemical or optical transduction methods as shown in **Fig. 4**.

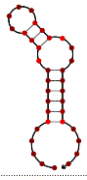
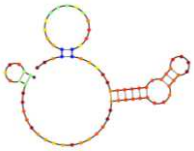
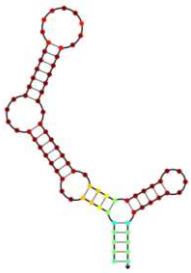
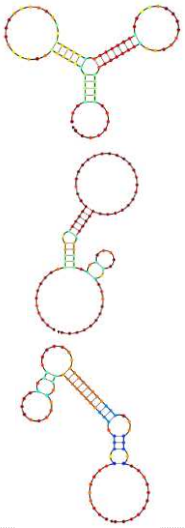
Fig. 4a exhibits the collected approaches presenting results on electrochemical (48%) or optical (50 %) development of aptasensing strategies, which are deeply detailed in the right pie chart of **Fig. 4a.** Of all works revised in this review, only the 9 % focus on characterizing and studying the affinity and binding properties (providing the affinity constant K_d) of the selected aptamers for E2 (Akki et al., 2015; Svobodová et al., 2017; Vanschoenbeek et al., 2015), and P4 (Skouridou et al., 2017). Importantly, **Fig. 4b** exhibits the most common biological fluids and other matrices used in all studies included in this review.

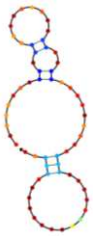
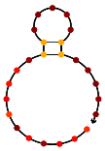
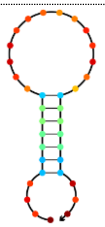
Table 2 provides a list of aptamer sequences used in published studies discussed within this review. It is important to bear in mind that some authors make use of the same (previously published) sequences but with a slight modification (e.g., length by shortening or splitting the aptamers). However, **Table 2** only shows the original sequence on which the linked studies were based and its corresponding two-dimensional structure making use of the Nucleic Acid Package (NUPACK) online software ("http://www.nupack.org/," 2022; Yu et al., 2021). Importantly, these structures are not necessarily accurate without further experimental characterization (Miller et al., 2022). In some cases, the sequences are highlighted in different parts showing the shared regions (letters in blue and italics) of the aptamers depending on the length utilized and the variations (orange and bold with respect to the first sequence of that raw) within one aptamer when several sequences were studied within the same manuscript. In the case of aptamers used for the specific recognition of E2, there are mainly three options in terms of length, the 35-mer, 75-mer, and 76-mer aptamers. As it is possible to see in the first two rows of the second column, the shorter version (35-mer, originally generated and characterized by Alsager *et al.* (Alsager et al., 2015)) is the same sequence as the center part of the 75-mer aptamer. Specific identifiers (ID) have been assigned for a better understanding of the aptamer used in each case.

On the other hand, the 76-mer aptamer (i.e., E2-76) is a completely different sequence of oligonucleotides and it does not match with any other region of the other two options. Importantly, several variations of these three aptamers represented in **Table 2** have been employed for the development of truncated, split versions of those aptamers. Moreover, a fourth raw shows variations depending on different SELEX procedures (showing only the three aptamers with the highest selectivity in **Table 2**). For the case of P4 aptamers, there are three different rows with multiple options and variations but the two first ones are also closely related since the shorter sequence (25-mer, P4-25) is contained in part of the larger and more

commonly used one (P4-60). A justification for shortening the original sequence (P4-60) was provided by Alhadrami and coworkers (Alhadrami et al., 2017).

Table 2. Aptamer sequences utilized within the reviewed works for estradiol (E2), progesterone (P4), luteinizing hormone (LH), and follicle-stimulating hormone (FSH).

Target / ID	Aptamer sequence	Structure NUPACK	Ref.
E2 (35-mer, contained in 75-mer) / E2-35	5'-*AAGGGATGCCGTTTGGGCCCAAGTTCGGCATAGTG*-3'		(Alsager et al., 2017, 2015; Chang and Yeh, 2021; Eisold and Labudde, 2018; Huang et al., 2016; Qi et al., 2018; Zheng et al., 2015)
E2 (75-mer) / E2-75	5'-ATACGAGCTTGTTCAATACG*AAGGGATGCCGTTTGGGC CCAAGTTCGGCATAGTG*TGGTGATAGTAAGAGCAATC-3'		(Alsager et al., 2014; Qi et al., 2018; Zheng et al., 2015)
E2 (76-mer) / E2-76	5'-GCTTCCAGCTTATTGAATTACACGCAGAGGGTAGCGGC TCTGCGCATTCAATTGCTGCGCGCTGAAGCGCGGAAGC-3'		(Alsager et al., 2014; Dong Huy et al., 2011; Du et al., 2017; Fan et al., 2015; Huang et al., 2015, 2014b, 2014a, 2021; Ke et al., 2014; Kim et al., 2007; Liu et al., 2014; M. Liu et al., 2019; X. Liu et al., 2019; Nameghi et al., 2019; Ni et al., 2017; Olowu et al., 2010; Qiao et al., 2021; Yang et al., 2019; Yildirim et al., 2012; Zhang et al., 2016; Zhu et al., 2015)
E2 / (6A, 1B, and 19B)	5'-AGCAGCACAGAGGTCAGTTCGTCGAATCAGCACCTCTGCATAGGT TACGTTTATACTGCGCCTATGCGTGCTACCGTGAA-3' 5'-TGTGTGTGAGACTTCGTTCCGGCGATGGGGTAGGGGGTGTGG AGGGGCCGGACGGAGGGGCAGCAAGGCATCAGAGGTAT-3' 5'-TGTGTGTGAGACTTCGTTCCCCCGGTCGGTGGGGTAGGGGGCGTG GAGTCACCGGGGGGGCAGCAAGGCATCAGAGGTAT-3'		(Vanschoenbeek et al., 2015)

P4 (60-mer) / P4-60	5'-GCATCACACACCGATACTCACCCGCCT*GATTAAC ATTAGCCCACCGCCCACC*CCCCGCTGC-3'		(Alhadrami et al., 2017; Alnajrani and Alsager, 2019; Contreras Jiménez et al., 2015; Du et al., 2016a, 2016b; Samie and Arvand, 2020; Skouridou et al., 2017)
P4 (25-mer contained on 60-mer) / P4-25	5'-*GATTAACATTAGCCCACCGCCCACC*-3'		(Cui et al., 2021; Zhu et al., 2020)
P4 / P4-diff	5'-(T_7)CTAGAGA**T**GTYTGTCTCAACGTGCCCTG**CG**WCGTGATC(T_5)-3' 5'-(T_7)CTAGAGA**A**GTYTGTCT**TTCGCAY**C**AAGAGWTACG**GATC(T_5)-3' 5'-(T_7)CTAG**CTTCCGTGAAAY**CAACGTGCCCTG**GW**WCGT**CTAG**(T_5)-3'	---	(Zeidan et al., 2016)
LH (35-mer) / LH-35	5'-TATGGTATGCTGTGTGGTATGGGGTGGCGTGCTCT-3'		(Sun et al., 2021)
LH (35-mer) / LH-35*	5'-TGTGGTGGTGGTTGGGGGTGGTGGGTGGGATGGTA-3' 5'-TGTGGTGGTGGTTGGGGGTGGTGG**A**TGGGATGGTA-3' 5'-TGTGGTGGTGGTTGGGGGTGGTGGGTGGGATGG**AA**-3'	---	(Liang et al., 2019)
FSH-R (37-mer) / FSH-R-37	5'-TGGGCACTATTTATATCAAC(N_{40})AATGTCGTTGGTGGCCC-3'	---	(Ibrahim, 2013)

***** Note: sequences contained in longer versions of the aptamers are written in blue and italics and differences among the sequences in each row are highlighted in bold and orange.

3.1. Estradiol

Steroid hormones are a family of small hydrophobic molecules (**Fig. 1c**) derived from cholesterol that has been targeted by many clinical assays using a variety of bodily fluids with very low target concentrations (Wheeler, 2013). The small molecule 17β-estradiol (here represented as E2) is the most active and potent type of estrogen that plays a role in ***i)*** regulating estrous and menstruation cycles in females, ***ii)*** the development of secondary sexual characteristics (Huang et al., 2016), ***iii)*** maintaining female reproductive tissues (Huang et al., 2021), low levels prevent ovulation and result in difficulties in becoming pregnant (Thilagam et al., 2014), ***iv)*** the ovarian system since it affects the proliferation and formation of gap junction connections, including stimulating uterine growth, maturing germ cells, and maintaining a healthy pregnancy (Chang and Yeh, 2021; Huang et al., 2021), ***v)*** reducing blood cholesterol by regulating the distribution of body fat, and ***vi)*** in preventing bone loss by maintaining bone and joint health (Chang and Yeh, 2021; Ibrahim, 2013).

Since E2 levels vary throughout women's life and due to the aforementioned roles, the quantification of estrogen and/or E2 levels in biological samples (e.g., serum or urine) is important in various clinical evaluations including investigations of fertility treatments, post-

menopausal status, pregnancy, hyperandrogenism, and/or different cancers (Huang et al., 2016, 2021; Kuramitz et al., 2003). More specifically, high concentrations of E2 are associated with increased breast cancer risk and may cause liver damage (Chang and Yeh, 2021). In general, it is also important to remark that human exposure to even low concentrations (<300 nM in water samples) of estrogen can disorganize the activity of the endocrine system and lead to different cancers (Nameghi et al., 2019; Zhang et al., 2016). Additionally, previous studies have reported that E2 can negatively influence the proliferation and migration of newborn cells (Giannoni et al., 2011). Hence, the ingestion of residues of E2 present in animal products such as milk can promote harmful effects in humans, therefore becoming a global concern for the scientific community (Chang and Yeh, 2021).

The widely lab-bench-employed analytical methods for estrogen and/or E2 detection are based on immunoassay and instrumental analytical approaches, like HPLC, and liquid chromatography-mass spectrometry (LC-MS). Most of these analytical approaches have in common the need for complex pretreatments and highly-skilled personnel. Furthermore, most of these approaches are time-consuming and produce organic solvent wastes that are dangerous to the environment (Huang et al., 2016; Nameghi et al., 2019; Rather et al., 2018). Therefore, there is a strong demand for the development of a rapid, sensitive, and specific method to detect and quantify E2 in all types of environmental, foodstuffs, and clinical samples to preserve the environment and the health of human beings (Chang and Yeh, 2021; Nameghi et al., 2019).

Because E2 is considered the most potent and active estrogen, the number of literature aptamer-based studies is larger in comparison with other fertility-related hormones. In this review, **Table 3** and **Table 4** summarize the electrochemical and optical aptasensing approaches, respectively, centered on the recognition of this steroid for environmental control and endocrine disruptive measures.

Regarding **Table 3** mostly, voltammetric approaches using glassy carbon electrodes (GCEs) modified with nanomaterials (typically AuNPs) and/or other nanocomposites such as vanadium disulfide nanoflowers,(Huang et al., 2014a) tungsten disulfide nanosheets,(Huang et al., 2014b) cobalt sulfide nanosheets-AuNPs film (Huang et al., 2015) or carbon nanotubes composites (X. Liu et al., 2019) have been developed not only for the detection of E2 in environmental, biological and pharmacological samples (**Fig. 4b**) (Rather et al., 2018). The use of conducting polymers (e.g., pyrrole) is scarce but is an option for the recognition of E2 in biological samples showing wider linear ranges and outstanding limits of detection (LODs) in the low fM range (Zhu et al., 2015). From a point of view more focused on the aptamer behavior, it is important to highlight that normally, the voltammetric sensing mechanisms depend on the aptamer-E2 interaction, enhancing or hindering the electron transfer of the

redox reporter. For instance, Kim *et al.* showed that the current decreased upon the interaction of E2 with the DNA aptamer due to the interference of E2 with the electron flow produced by a redox reaction between ferro- and ferricyanide at the electrode surface (Kim et al., 2007). In the case of Ke and coworkers, the interfacial electron transfer resistance increased significantly after E2 interaction with the aptamer, which led to the increment of the electrochemical (in this specific case impedimetric) signal which can be correlated with the E2 concentration (Ke et al., 2014).

Another commonly used option is the employment of gold electrodes for the direct self-assembly of a thiolated E2 aptamer at the surface via Au-S bonding and passivation with 6-mercapto-1-hexanol (MCH) (Fan et al., 2015; M. Liu et al., 2019; Nameghi et al., 2019). The specific schematization of the protocol followed by Fan *et al*. is summarized in **Fig. 5a**. Interestingly, the approach of Nameghi *et al.* using a thiolated-modified split version of the E2-76 aptamer (**Table 2**) significantly prohibits the access of the redox probe (ferro- and ferricyanide) to the gold electrode surface in presence of the target, leading to a weak current (Nameghi et al., 2019) as can be seen in **Fig. 5b**. This strategy is being increasingly explored by different groups centered on optical approaches (**Table 4**) since it highlights interesting improvements compared to using longer aptamers. Apart from those voltammetric examples, we can highlight the photoelectrochemical (PEC) approach suggested by Fan and coworkers (**Fig. 5c**) in which the PEC aptasensing strategy uses cadmium selenide nanoparticles modified with titania (TiO_2) nanotube arrays to reach an outstanding LOD in the low fM range (Fan et al., 2014). However, cadmium is a highly toxic carcinogen harmful to most of the body's systems (organs, including the reproductive system), limiting its use in clinical settings (Rahimzadeh et al., 2017). Interestingly, PEC approaches suggested by Du *et al.* (based on the use of ITO hematite/N-doped graphene film) and Feng *et al.* (employing molybdenum-doped porous bismuth vanadate (Mo-p $BiVO_4$) nanoarray) take advantage of different nanostructures to increase the number of binding sites to immobilize more aptamers and decrease LODs (Du et al., 2017; Feng et al., 2020). Label-free impedimetric gold electrodes (Lin et al., 2012) and CNT-based field-effect transistor (Zheng et al., 2015) approaches toward E2 detection in human urine or buffer, respectively, are also reported. In recent years, there is an apparent trend in using aptamers linked to nanomaterials to maximize binding sites and decrease the LOD to clinically relevant levels in biofluids.

Table 3. Electrochemical (differentiating between CV, SWV, EIS, DPV, and FET) and photoelectrochemical (PEC) aptamer-based sensing approaches for 17β-estradiol (E2).

Electrode (sensing type)	Aptasensing system & modifications	Aptamer Used (ID)	Linear Range	LOD	Sample	Recovery (%) / RSD (%)	Ref.

ITO hematite/ (PEC)N-doped graphene film	α-Fe_2O_3 nanocrystals and NG with AuNRs	E2-76	1 fM–1 nM	0.33 fM	Milk powder	86.96–102.94 / 2.3~7.4	(Du et al., 2017)
ITO (PEC)	Mo-doped porous $BiVO_4$/Bi_2S_3 array > aptamer binding sites.	---	1 fM–1 nM	0.32 fM	---	97.6–104.9 / 1.1 – 3.6	(Feng et al., 2020)
Au-electrode (DPV)	NiHCF NPs covered with AuNPs + self-assembly aptamer with MCH	E2-76	1 pM–0.6 nM	0.8 pM	Municipal wastewater	93.6–100.2 / 1.1 – 3.6	(Fan et al., 2015)
Au-electrode (PEC)	TiO_2 NTs modified with CdSe NPs	E2-76	0.05–15 pM	33 fM	Medical wastewater, lake water & tap water	90.0–102.8 / < 5.45	(Fan et al., 2014)
GCE (DPV)	Vanadium disulfide nanoflowers and AuNPs	E2-76	10 pM–10 nM	1 pM	Urine samples	92.0–105.2	(Huang et al., 2014a)
GCE (DPV)	WS_2 nanosheets + AuNPs (via Au–S)	E2-76	10 pM–5 nM	2 pM	Serum and water	96.0–104.2 / 1.8 – 3.2	(Huang et al., 2014b)
GCE (DPV)	CoS/AuNPs film + assembly thiol aptamer + MB-complementary sequence	E2-76	1 pM–1 nM	0.7 pM	Urine samples	94.4 – 104 / 1.6–3.4	(Huang et al., 2015)
BDD electrode (EIS)	Hierarchical dendritic gold microstructure on BDD	E2-76	10 fM–1 nM	5 fM	Real water samples	98.3–105.7 / 4.2–9.3	(Ke et al., 2014)
Au-electrode chips (SWV)	Avidin-biotin immobilization	E2-76	1–0.01 nM	0.1 nM	Buffer	--- / 7	(Kim et al., 2007)
Au-electrode (DPV)	MCH self-assembled monolayer + graphene	E2-76	0.07–10 pM	50 fM	Spiked real water	89–115.9 / 1.1–5.1	(M. Liu et al., 2019)
CNT-FET (FET)	Liquid gated CNT FETs + aptamer immobilization (EDC/NHS)	E2-35 E2-75	50 nM–1.6 µM	50 nM	Buffer	--- / ---	(Zheng et al., 2015)
GCE (EIS)	Aptamer-functionalized nanoporous ECP	E2-76	~1 fM–1.6 µM	1 fM	Spike tap and urine samples	--- / ---	(Zhu et al., 2015)
Au-electrode (EIS)	Label-free EIS aptamer (via Au-S)	E2-76	10 nM–10 pM	2 pM	Human urine	91.7–101.3 / 3.2–4.4	(Lin et al., 2012)
Au-electrode (CV and SWV)	PEDOT doped with AuNPs + biotinylated aptamer	E2-76	0.1–100 nM	0.02 nM	Buffer	--- / ---	(Olowu et al., 2010)
Au-electrode (DPV)	Thiol-modified split aptamers immobilized (via Au-S)	E2-76	1.5–100 & 100–7000 pM 3–300 & 300–9000 nM	0.5 pM 0.7 pM	Tap water, spike milk and serum samples	--- / --- 89.4–101.7 / 1.8–6.4 92.1–102 / 3.9–6.7	(Nameghi et al., 2019)
GCE (DPV)	AuNP-Thi-CNTs nanocomposite (passivation with MCH)	E2-76	12 pM–60 nM	1.5 pM	Spike diluted serum samples	94.6–107.6 / 7.83	(X. Liu et al., 2019)
GCE (SWV)	NH_2-AptE2 on ERGO/GCE and surface-functionalized diazonium salts	---	1 fM–9 pM 12pM–23nM	5 fM	Spike pharma dosages	94.05–100.47 / 1.2–3.3	(Rather et al., 2018)

ITO: indium tin oxide; PEC: photoelectrochemical; α-Fe_2O_3: hematite, NG: N-doped graphene, AuNRs: gold nanorods; CV: cyclic voltammetry; SWV: square wave voltammetry; EIS: electrochemical impedance spectroscopy; DPV: differential pulse voltammetry; Mo: molybdenum, $BiVO_4$: bismuth vanadate, Bi_2S_3: bismuth sulfide, NiHCF NPs: nickel hexacyanoferrate nanoparticles; AuNPs: gold nanoparticles; MCH: 6-mercapto-1-hexanol; PEDOT: poly(3,4-ethylenedioxythiophene); TiO_2 NTs modified with CdSe NPs: titanium dioxide nanotubes modified with cadmium selenide nanoparticles; GCE: glassy carbon electrode; WS_2 nanosheets: layered tungsten disulfide; CoS/AuNPs film: two-dimensional cobalt sulfide nanosheet/gold nanoparticles film; MB: methylene blue; BDD: boron-doped diamond; CNT FET: carbon nanotube field-effect transistors; EDC/NHS: N- (3-dimethylaminopropyl)N-ethylcarbodiimide hydrochloride/ N-hydroxysuccinimide; ECP: electrochemically conducting polymers; AuNP-Thi-CNTs nanocomposite: gold nanoparticles placed on thionine-multiwalled carbon nanotube; NH_2-AptE2: amine-functionalized estradiol aptamer; ERGO/GCE: electrochemical reduction of graphene oxide on glassy carbon electrode.

It is interesting to highlight here that most of the optical approaches (**Table 4**) are conducted in solution, hence, only a few examples can be described where the aptamer is immobilized. For example, Yildirim *et al.* covalently immobilized the E2-76 aptamer in an optical fiber via bovine serum albumin. With an indirect competitive detection mode, the samples containing different concentrations of E2 were premixed with a given concentration of fluorescence-labeled DNA aptamer, which highly specifically binds to the hormone. Then, a higher concentration of E2 leads to less fluorescence-labeled DNA aptamer bound to the sensor surface and thus to a lower fluorescence signal (Yildirim et al., 2012). Another case is followed

by Huang and coworkers immobilizing the same aptamer on an amine-modified polystyrene ELISA surface which allows the hormone to interact with and be sandwiched by antibodies (Huang et al., 2021), as is specifically exhibited in **Fig. 5d**. Other types of attachment exist such as the example of the E2-76 aptamer anchored to isothiocyanate-modified glass beads. But in this case, the authors focused more on developing a separation and enrichment method of the E2 aptamer rather than developing an optical sensor for E2 recognition (Dong Huy et al., 2011).

As previously explained in **Section 2.3**, colorimetric assays based on citrate-capped AuNPs are a widely used tool to investigate aptamer−target interactions since it likely provides a fast method to test aptamer performance (Bottari et al., 2020). Zhang and coworkers, however, presented a simple colorimetric label-free aptasensing approach in which the formation of the aptamer-E2 complex promotes poly(diallyldimethylammonium chloride) aggregation with AuNPs causing a remarkable color change (Zhang et al., 2016). Moreover, as AuNPs have quenching properties, they are suitable for fluorometric aptasensors (Alsager et al., 2015; Qi et al., 2018). In the same line, quantum dots (QDs) are used as quenchers labeled to a complementary oligonucleotide that binds to the aptamer when the target is absent. Upon target binding, the quencher-labeled complementary oligonucleotide dissociates, and the fluorescence signal increases (**Fig. 5e**). In the example published by Huang and coworkers (**Fig. 5f**), the authors used a similar approach using a ruthenium complex (Ru) which quenches the fluorescence of QDs when the target is present since Ru has a greater affinity to binding with free aptamers (Huang et al., 2016). Similarly, Ni *et al.* developed a fluorescent approach using label-free aptamers and rhodamine B as fluorescent label quenched by AuNPs (Ni et al., 2017).

Table 4. Optical aptamer-based sensing approaches for 17β-estradiol (E2).

Electrode (readout)	Aptasensing system & modifications	Aptamer used (ID)	Linear Range	LOD	Sample	Recovery (%) / RSD (%)	Ref.
Au-electrode (Fluorescent)	Aptamer tethered to the surface of carboxylated polystyrene NPs	E2-75 E2-76	5–150 nM	5 nM	Buffer	--- / ---	(Alsager et al., 2014)
GCE (Colorimetric)	Aptamer-coated AuNPs	E2-35	5–400 nM 50–800 nM	5 nM 200 pM	River and spiked rat urine	--- / ---	(Alsager et al., 2015)
In solution (Colorimetric)	aptamer-E2 complex and aggregation of AuNPs by PDDA	E2-76	1.57–350 nM	1.57 nM	Water samples	104.5–114.5 2.79–4.34	(Zhang et al., 2016)
In solution (Fluorescent)	Label-free aptamer + AuNPs and RhoB (aggregation of AuNPs)	E2-76	0.48–200 nM	0.48 nM	Spiked water samples	94.3–111.7	(Ni et al., 2017)
FOCA (CHML)	fiber-optic biosensor + Si-based photodiode detector	E2-76	1.17–411.9 nM	0.18 nM	Tap, bottled, wastewater treatment plant	80.3–105.7 / < 9.2	(Yang et al., 2019)
In solution (Fluorescent)	Ru complex and quantum dots (QDs) as fluorescence probes	E2-35	0.08–0.4 μM	37 nM	Diluted fetal bovine serum	96.5–104.8 / 2.2–10.1	(Huang et al., 2016)
In solution (Colorimetric)	AuNPs aggregation principle using a split aptamer (P1+P2)	E2-76	0.37 nM–367 μM	0.37 nM	---	--- / ---	(Liu et al., 2014)

Optical fiber (Fluorescent)	Fluorophore-labeled aptamer immobilized via BSA	E2-76	0.7–11 nM	2.1 nM	Spiked wastewater effluents	94.1–105.3 / ---	(Yildirim et al., 2012)
In solution (Colorimetric)	Split aptamer better aggregation of AuNPs	E2-35	6.7 nM–66.7 μM	6.7 nM	Synthetic urine samples	--- / ---	(Chang and Yeh, 2021)
In solution (Colorimetric)	Truncated aptamer (Kim2007) + aggregation AuNPs	E2-76	73–36700 nM	73 nM	Water and milk	96.2–114.4 / 2.3–11.9	(Qiao et al., 2021)
Apt–E2–Ab sandwich (ELISA)	Apt-CNP + Apt-GNP Ab immobilized on amine-modified polystyrene ELISA surface	E2-76	1.5–50 nM	1.5 nM	Spiked human serum	--- / ---	(Huang et al., 2021)
FRET-based sensor (Fluorescent)	AuNPs as fluorescent nanoquencher	E2-35 E2-75	0–73 nM	3.7 nM	Spike real water	99.7–102.9 / 0.3–2.91	(Qi et al., 2018)
LFA (Colorimetric)	Aptamer + AuNPs, negative BSA, and positive Lys	E2-35	50–1000 nM	50 nM	spiked river water samples	--- / ---	(Alsager et al., 2017)

GCE: glassy carbon electrode; AuNPs: gold nanoparticles; PDDA: poly(diallyldimethylammonium chloride); RhoB: rhodamine B; FOCA: fiber optic chemiluminescent aptasensor; CHML: chemiluminescence; Ru: ruthenium; QDs: quantum dots; BSA: bovine serum albumin; NCS beads: isothiocyanate-modified beads; Apt-E2-Ab: Aptamer–17β-estradiol–antibody sandwich; ELISA: enzyme-linked immunosorbent assay; Apt-CNP/GNP: carbon/gold nanoparticle-conjugated aptamer; FRET: Fluorescence Resonance Energy Transfer; LFA: lateral flow assay; Lys: lysozyme.

As was mentioned, AuNPs in combination with aptamers have been extensively employed for different applications since nanoparticle-based colorimetric aptasensors allow for simple and direct detection of several target molecules (Alsager et al., 2018; Lee et al., 2019). Nevertheless, several DNA aptamers with specific secondary structures, such as the E2 aptamer, cannot electrostatically stabilize AuNPs, which causes aggregation of the AuNP and complicates colorimetric detection, affecting the sensitivity and analytical performance of the aptasensor (Chang and Yeh, 2021). Therefore, a new trend using split aptamers to overcome this limitation emerged (working mechanism shown in **Fig. 5b**). The principle suggests that aptamers can be split into two fragments that remain separate in the absence of the target but assemble in its presence (Chang and Yeh, 2021). Such split aptamers retain their affinity and specificity and enhance the sensitivity of an assay (Luo et al., 2019; Morris et al., 2018). Chang and Yeh proposed a sensitive colorimetric biosensor for label-free E2 detection using an E2-specific split aptamer-based on the 35-mer sequence shown in **Table 2**. Their results showed a higher sensitivity when a split aptamer with high free energy of the structure ($\Delta G > -3$ kcal/mol) is used compared to one with low free energy of the structure ($\Delta G < -3$ kcal/mol) at 27 °C (Chang and Yeh, 2021). This result supports the development of a highly sensitive AuNP-based colorimetric aptasensor by using a split aptamer-based biosensor after investigating the sensitivity of several structures with different free energies (Chang and Yeh, 2021).

Liu and coworkers performed a similar study, in which the E2-76 aptamer was divided into two short structures (34-mer and 33-mer) to improve the sensitivity of the AuNP-based colorimetric assay. Their results showed that the split aptamer retained the original 76-mer aptamer's affinity and specificity but increased the LOD 10-fold (Liu et al., 2014). Likewise, Namegui *et*

al. and Liu *et al.* made use of the same split1-E2-split2 complex assembled on the electrode surface in order to study the chronic exposure to E2 by measuring E2 levels in tap water and milk samples, respectively (Liu et al., 2014; Nameghi et al., 2019).

Another interesting work published by Alsager and coworkers focused on comparing a long aptamer (E2-75) and shorter variants (35-mer and 22-mer). Following an examination of the two-dimensional structure of the E2-75 aptamer for E2 that the authors previously reported (Alsager et al., 2014). Alsager *et al.* found that simply eliminating 20 to 30 nucleotides on either side of a core sequence yields a 25-fold improvement in LOD (from 5 nM to 200 pM) along with improved discrimination against structurally similar molecules. The improved sensitivity with the 35-mer and 22-mer aptamers is only partially related to a higher affinity for E2 (K_d ~14 and 11 nM, compared to ~25 nM for the E2-75 aptamer). By using surface-sensitive electrochemical techniques, the authors found that the target-bound E2-75 aptamer has some affinity for AuNPs which directly results in a suppressed colorimetric signal. By comparing the 35-mer with the minimalistic 22-mer, the 35-mer aptamer was selected as the optimal one since it ensures the balance between binding to the AuNPs and E2 (Alsager et al., 2015).

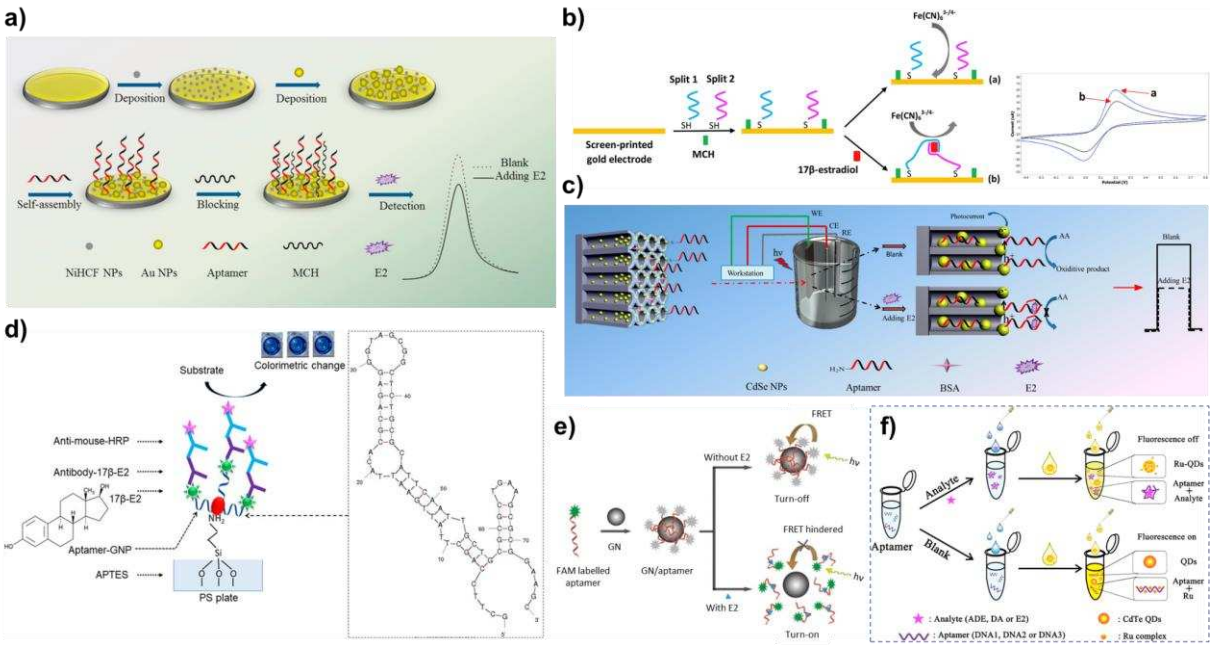


Fig. 5. Schematic overview of some discussed electrochemical and optical aptamer-based sensing approaches for 17β-estradiol (E2). Reproduced with permission of **a)** (Fan et al., 2015), Copyright (2015), Elsevier; **b)** (Nameghi et al., 2019), Copyright (2019), Elsevier; **c)** (Fan et al., 2014), Copyright (2014), American Chemical Society; **d)** (Huang et al., 2021), Copyright (2020), Wiley-VCH; **e)** (Qi et al., 2018), Copyright (2018), Royal Society of Chemistry; and, **f)** (Huang et al., 2016), Copyright (2016), Elsevier.

3.2. Progesterone

Much like E2, P4 serves as an indicator of early pregnancy and participates in controlling hormonal levels throughout the reproductive cycle (Zeidan et al., 2016). It is an endogenous steroid hormone that is produced by the adrenal cortex and gonads, being secreted from the *corpus luteum* (**Fig. 1**). P4 is mainly associated with the regulation of mammalian pregnancy, animal growth, and development (Alhadrami et al., 2017; Velayudham et al., 2021). It is also used as a therapeutic drug in menopause, maintaining pregnancy, and as an oral contraceptive (Filicori, 2015). Due to its central roles in biology and medicine, the development of methodologies for the sensitive detection of P4 in diverse matrices became increasingly important (Ehrentreich-Förster et al., 2003; Velayudham et al., 2021).

Healthy P4 concentrations vary through the menstrual cycle and are increased during pregnancy (more details in **Table 1**) (Alhadrami et al., 2017; Zeidan et al., 2016) but if these healthy concentrations are exceeded, many side effects can occur, including headache, weight gain, breast discomfort, urinal infections, mental depression, among others (Sherwin, 1999). Moreover, epidemiological studies have confirmed that the disbalance of P4 levels increases the chance of breast and cervical cancer (Key et al., 2011). From the exposure point of view, it is important to highlight that the consumption of high levels of P4, found for instance in cow's milk, may also promote breast and lung cancers (Club, 2010; Gallus et al., 2006). Especially, milk contains a considerable amount of both estrogen and P4 concentrated in fat (Cui et al., 2021; Roelofs et al., 2006). Therefore, after a high amount of P4 is consumed, the body retains a certain quantity but the rest is released into the environment. Hence, the impact that female hormones in food have on human health (not only on women) and the environment is tremendous. Consequently, monitoring P4 levels in both environmental and clinical samples is highly important (Alhadrami et al., 2017).

The most commonly used methods for the detection of P4 in different matrices (i.e., human biological and clinical samples) have historically been immunological approaches such as ELISA (Samsonova et al., 2018) and RIA (Velayudham et al., 2021), electrophoresis-chemiluminescence detection (Ye et al., 2013), HPLC (Decheng et al., 2021), fluorescence spectrophotometry (Tschmelak et al., 2005), and non-competitive idiometric or fluoroimmunoassays (Barnard et al., 1995). However, despite providing sensitive measurements, as generally mentioned in the first part of this review, these laboratory tests still require sophisticated analytical facilities and demand very carefully controlled and time-consuming procedures, skilled experts to perform the analysis, and labeled probes to detect the steroid. Overall, such drawbacks render the techniques unsuitable for large-scale, or most importantly, decentralized detection (Cui et al., 2021; Zeidan et al., 2016). Furthermore, a major drawback of these clinical lab-bench tests is the high levels of interference arising from

the structural similarities among different steroid hormones (Zeidan et al., 2016). In this context, electrochemical and optical sensors for monitoring steroid hormones are receiving much attention because of their robustness and cost-efficient instrumentation designs (Manickam et al., 2018; Velayudham et al., 2021).

In recent years, different types of sensor architectures involving aptamers have been reported for P4 detection (**Table 5, Fig. 6**). Out of the 10 publications (presenting aptamer-based sensors for P4 detection) found in the literature, 4 are based on electrochemical approaches, and 6 on optical methods. It is worth mentioning the variety of electrochemical approaches (from impedimetric-based and voltammetric gold electrodes) with the self-assembled P4 aptamers for the monitoring of P4 in environmental (Contreras Jiménez et al., 2015) and diluted blood samples (Velayudham et al., 2021), respectively, reaching LODs in the low nM range. Interestingly, the first example followed by Contreras Jiménez and coworkers suggested an impedimetric aptasensor based on the conformational change of the aptamer, immobilized on the gold electrode by self-assembly, that results in an increase in electron transfer resistance upon binding to P4 (**Fig. 6a**). Regarding the second case, Velayudham *et al.* used hydrogels in which the aptamer was favorably integrated into the internal pores and interconnected inside the networks created in that type of matrix (**Fig. 6b**). The hydrophilic nature of the hydrogel network acts as a blocking membrane that helps in minimizing the non-specific binding of the voltammetric biosensor (Velayudham et al., 2021).

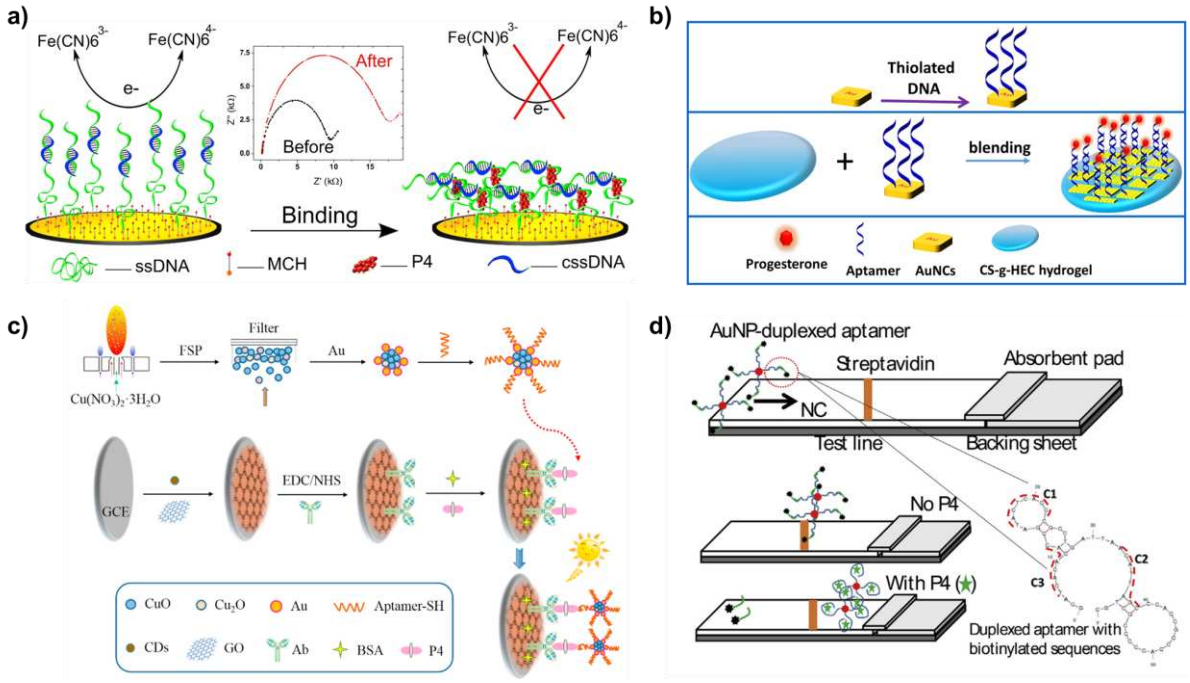


Fig. 6. Schematic summary of a few of the discussed electrochemical and optical aptamer-based approaches for Progesterone (P4) detection. Reproduced with permission of **a)** (Contreras Jiménez et al., 2015), Copyright (2015),

American Chemical Society; **b)** (Velayudham et al., 2021), Copyright (2021), Elsevier; **c)** (Zhu et al., 2020), Copyright (2020), Elsevier; and **d)** (Alnajrani and Alsager, 2019), Copyright (2019), Elsevier.

Other examples pursue the same goal by using SPCEs or GCEs in combination with graphene, QDs, and gold nanostructures such as gold nanoflakes or gold-copper oxides for electrochemical (Samie and Arvand, 2020) or photoelectrochemical (Zhu et al., 2020) readouts, respectively. In the first case, the label-free approach proposed by Samie and Arvand, the authors used the 60-mer aptamer-P4 complex formation to hinder the electron transfer reaction on the sensing interface (Samie and Arvand, 2020). In the second example, Zhu and coworkers presented a novel hybrid antibody-aptamer (P4-25) sandwich-based sensor (**Fig. 6c**) with a photoelectrochemical readout (Zhu et al., 2020). The cathodic photocurrent of the biosensor increases linearly with P4 in a wide concentration range (0.5 – 180 nM) and is able to reach extremely LODs (0.17 nM) demonstrating high specificity, reproducibility, and excellent stability of the hybrid sensor for P4 detection in human serum samples (Zhu et al., 2020).

On the other hand, the optical approaches are mainly based on the colorimetric response given by the aggregation of AuNPs in solution (Du et al., 2016b, 2016a) or lateral flow assay (LFA) as **Fig. 6d** exhibits (Alnajrani and Alsager, 2019). LFAs are easy-to-use and avoid the need to identify the optimal salt concentration to trigger the color change, thus offering a benefit for real samples (Alsager et al., 2017). Moreover, different quenchers can be used for fluorescent sensing either ***i)*** via carbon dots and graphene oxide quenching (Cui et al., 2021) or ***ii)*** through a fluorescence-quencher pair assay using a complementary labeled Dabcyl and a truncated P4 aptamer (P4-60, **Table 2**) approach (Alhadrami et al., 2017). Finally, nano-enhanced surface plasmon resonance imaging (SPRi)-based sensor has been developed by Zeidan *et al*. (Zeidan et al., 2016). Here, the authors took advantage of the multi-array feature of SPRi to construct a biosensor to screen nine next-generation aptamers (X-aptamers) simultaneously. X-aptamers are a new generation of aptamers designed to improve the binding and versatility of regular DNA/RNA-based aptamers (He et al., 2012). The developed biosensor can identify the binding capabilities of each aptamer (the three ones with the highest affinity are shown in **Table 2**). Importantly, the authors characterized the aptamer and its binding capability before employing it as a bio-recognition element. Certainly, this approach is crucial to confirm the specificity of the aptamer towards the analyte of interest (Bottari et al., 2020; Cánovas et al., 2022; Daems et al., 2021). The reason being is mainly due to the high number of aptamers used and suggested in literature in contrast with the lack of applied aptamers with robust sensitivity, specificity, and selectivity. Hence, complementary

characterization of the aptamer binding and its mechanism are required when proposing new biosensing strategies.

Table 5. Aptamer-based sensing approaches for progesterone (P4).

Electrode (sensing type)	Aptasensing system & modifications	Aptamer used (ID)	Linear Range	LOD	Sample	Recovery (%) / RSD (%)	Ref.
LFA (Optical)	AuNPs-60-mer aptamer and 8-mer complementary sequence modified with biotin	P4-60	5–50 nM	5 nM	Buffer and spike tap water	--- / ---	(Alnajrani and Alsager, 2019)
Au-electrode (EC-EIS)	Self-assemble aptamer (P4G13) + complementary sequence	P4-60	32–190 nM	2.86 nM	Spiked tap water samples	99.86 / ---	(Contreras Jiménez et al., 2015)
In solution (Colorimetric)	surfactant-induced aggregation of AuNPs (via CTAB)	P4-60	0.89–500 nM	0.89 nM	Human serum and urine	102.4–116.5 / 2.8–6.5 93.2–107.6 / 3.4–5.5	(Du et al., 2016a)
In solution (Colorimetric)	Aggregation of uncoated AuNPs in presence of NaCl	P4-60	2.6–1400 nM	2.6 nM	Spike tap water and urine	84.4–115.0 and 94.7–118.8	(Du et al., 2016b)
SPCE (DPV)	GQDs–NiO-AuNFs nano-architecture + f-MWCNTs	P4-60	0.01–1000 nM	1.86 pM	Human serum and pharma. formulations	--- / 0.6–0.28 & 97.9–102.84 / 1. –1.7	(Samie and Arvand, 2020)
GCE (PEC)	Sandwich of Ab+Apt. CDs-GO composite(Ab) + Au-CuO-Cu_2O	P4-25	0.5–180 nM	0.17 nM	Spike diluted serum samples	92.5–106.7 / < 3.2	(Zhu et al., 2020)
Thin metal-coated surface (SPRi)	Aptamer tagged with biotin-avidin	P4-diff	5 nM–400 µM	5 nM	Buffer	--- / ---	(Zeidan et al., 2016)
Au-electrode (SWV)	AuNCs + thiolated aptamer and CS-g-HEC hybrid hydrogel	---	3.2–320 nM	3.2 nM	Spike diluted blood	--- / ---	(Velayudham et al., 2021)
Fluorescence-quencher pair assay (Fluorescent)	tripartite dsDNA duplex + fluorescein and complementary labeled Dabcyl	P4-60	0–250 nM 32–320 nM	--- 0.35 nM	Spike tap water and artificial urine	88.6–95.2 / 9.35–12 87.2–97.3 / 8.23–11.6	(Alhadrami et al., 2017)
FRET system (Fluorescent)	Fluorescence based on CDs-GO quenching	P4-25	0.1–120 nM	33 pM	Buffer and milk	97.2–101.2 / 1.81 ---	(Cui et al., 2021)

LFA: lateral flow assay; EIS: Electrochemical Impedance Spectroscopy; AuNPs: gold nanoparticles; CTAB: cationic surfactant hexadecyltrimethylammonium bromide; NaCl: sodium chloride; SPCE: screen-printed carbon electrode; GQDs–NiO-AuNFs: graphene quantum dots nickel oxide gold nanoflakes; PEC: photoelectrochemical; fMWCNTs: functionalized multi-walled carbon nanotubes; MCH: 6-mercapto-1-hexanol; GCE: glassy carbon electrode; Ab+Apt: antibody and aptamer; CDs-GO: carbon dots and graphene oxide; AuNCs: gold nanocubes; SPRi: Surface Plasmon Resonance imaging; CS-g-HEC: chitosan and hydroxyethyl cellulose; FRET: fluorescence resonance energy transfer.

3.3. Gonadotropin-releasing hormone

Gonadotropin-releasing hormone (GnRH) is a decapeptide secreted into the hypophyseal portal system by specialized hypothalamic neurons, to reach its site of action at the pituitary gland. GnRH acts on pituitary gonadotropins to stimulate the production and secretion of gonadotropins LH and FSH (Hazum and Conn, 1988; Izzi-Engbeaya et al., 2020) which are inhibited afterward by negative feedback control by the reproductive steroids (i.e., E2 and P4) and inhibins (hormones secreted by the granulosa cells in the ovaries of women acting primarily to inhibit the secretion of FSH by the anterior pituitary gland) (Ibrahim, 2013; Keenan and Veldhuis, 2016) from the gonads (see **Fig. 1b**) (Wheeler, 2013). LH (**Section 3.3.1**) and

FSH (**Section 3.3.2**) stimulate the secretion of sex steroids from the gonads (primarily testosterone from the testes and estrogen and P4 from the ovaries) (Hazum and Conn, 1988). Apart from during the pre-ovulatory phase of the menstrual cycle, GnRH and LH secretion are inhibited by sex steroids (i.e., negative feedback) (Knobil et al., 1980; Wheeler, 2013). However, during the pre-ovulatory phase of the menstrual cycle, estrogen exerts positive feedback on GnRH and LH secretion, leading to increased LH secretion and the production of the LH surge required for ovulation (Izzi-Engbeaya et al., 2020; Knobil et al., 1980).

GnRH, LH, and FSH exhibit pulsatile secretory patterns, which are pivotal to their biological functions (Knobil et al., 1980), including the stimulation of gonadal sex steroid secretion. GnRH has a short half-life of 2–8 min in the peripheral circulation (Handelsman and Swerdloff, 1986), with very low levels (i.e., ≤ 4.12 pM) detected in plasma (Huseman and Kelch, 1978). Therefore, it is challenging to measure GnRH in the peripheral circulation (Izzi-Engbeaya et al., 2020). Besides, in order to monitor properly, peripheral blood sampling is necessary every 10 minutes for at least 8 h, increasing the cost of multiple analyses by immunochemical assays (e.g., ELISA) dramatically as has been previously discussed (Wheeler, 2013). As LH pulses are temporally coupled with GnRH pulses and LH is stable in blood (Clarke and Cummins, 1985), LH levels and pulses are often measured and used as the clinical gold standard surrogate markers of GnRH secretion (Izzi-Engbeaya et al., 2020).

3.3.1 Luteinizing Hormone

LH, also known as lutropin, is an interstitial cell-stimulating hormone playing a predominant role in the reproductive system and is highly correlated with infertility treatment (as clinical exogenous use) in both men and women (Sun et al., 2021). LH is a glycoprotein heterodimer (**Fig. 1c**) consisting of two subunits: α- and β-subunits (of 92 and 120 amino acids, respectively) (Padmanabhan et al., 2018) made by the pituitary gland. It plays a critical role in synthesizing the mature oocytes and in the downstream regulation of synthesizing sex steroid hormones (Liang et al., 2019; Sun et al., 2021). The secretion of LH is highly correlated with hypothalamic dysfunction, resulting in various reproductive disorders, such as hypothalamic amenorrhea and polycystic ovary syndrome (PCOS) (Coutinho and Kauffman, 2019). In healthy women, LH pulses occur every 1-2 h in the follicular phase and every 4 h in the luteal phase (Izzi-Engbeaya et al., 2020). When reproductive senescence occurs in menopausal women, LH levels are markedly elevated (resulting from the absence of negative feedback due to estrogen deficiency), and approximately one LH pulse occurs each hour (Prague et al., 2019). Women with hypothalamic amenorrhea have low LH levels and reduced LH pulsatility (Jayasena et al., 2014), whereas women with PCOS have high basal LH levels and increased

pulsatility (Coutinho and Kauffman, 2019; Izzi-Engbeaya et al., 2020). The LH plasma levels of healthy women in different stages of life and the ovarian cycle can be found in **Table 1**.

Currently, three major issues are preventing widespread clinical LH concentration profile measuring and its pulsatility analysis: ***i)*** there is no method able to monitor the pulsatility of LH in real-time since the LH concentration profile's resolution is restricted depending on the sampling protocol and the immunochemical assays; ***ii)*** the analysis cost (~€25 per sample, 50 samples per patient) when serial clinical chemiluminescent immunoassays are utilized; and ***iii)*** the analysis requires the use of advanced algorithms that can accurately take into account all variations regarding inherent biological stages, pulse-by-pulse, and physiological factors that might influence the secretion of the hormone and its elimination (Keenan and Veldhuis, 2016; Liang et al., 2019).

Therefore, there is a clear unmet medical need for translational technologies that could enable routine clinical LH pulsatility analysis for patients with reproductive disorders (Liang et al., 2019). Hence, identifying and quantifying the LH levels using an affordable and efficient method is crucial for diagnosing fertility, and gynecological endocrine-related and/or pituitary gland problems (Sun et al., 2021). However, quantifying the LH level remains challenging due to the inherent biological fluctuations, the impact of physiological factors on hormone secretion, and the analysis cost.

Table 6. Aptamer-based sensing approaches for luteinizing hormone (LH).

Electrode (sensing type)	Aptasensing system (modifications)	Aptamer used (ID)	Linear Range	LOD	Sample	Recovery (%) / RSD (%)	Ref.
Au wire electrode (SWV)	Reduced MB-modified B23 aptamer solution (thiolated + MCH)	LH-35*	5–500 nM & 1–500 nM	10.9, 10.7 nM	Spiked buffer & Serum	--- / ---	(Liang et al., 2019)
Interdigitated electrode (IDE)	GO-aptamer (via biotin-streptavidin)	LH-35	30 nM–1 µM	60 nM	Spiked diluted serum	--- / ---	(Sun et al., 2021)

Au wire: gold wire, MB: methylene blue, MCH: 6-mercapto-1-hexanol; IDE: interdigitated electrode; GO: graphene oxide.

Table 6 summarizes the only two publications found in the literature in which the authors develop an aptamer-based biosensor for LH detection. The two approaches used different aptamers and sensing mechanisms showing promising results in terms of sensitivity with LODs in the low nM range for spiked buffer and diluted serum samples. Liang *et al.* used a gold wire electrode set up with methylene blue (MB) as an electron transfer mediator (**Fig. 7a**). The thiolated aptamer is immobilized via Au-S bond on the gold surface which is later passivated with MCH. Recognition of LH by the aptamer induces a conformational change that alters the electron transfer rate between MB and the electrode allowing for the direct measurement of LH concentration (Liang et al., 2019). More recently, Sun and coworkers

designed an interdigitated electrode sensor (**Fig. 7b**) that is modified with graphene oxide (GO) to allow immobilization of the aptamer through a biotin-streptavidin interaction, which improves the current flow and enhances the electrochemical signal (Sun et al., 2021).

The work carried out by Leva *et al.* (Leva et al., 2002) has been previously grouped in other articles as a successful aptasensing approach toward LH hormone but it is important to remark that the authors were using Spiegelmers (i.e., RNA-like molecules built from L-ribose units) and for that reason that work has not been included in **Table 6**.

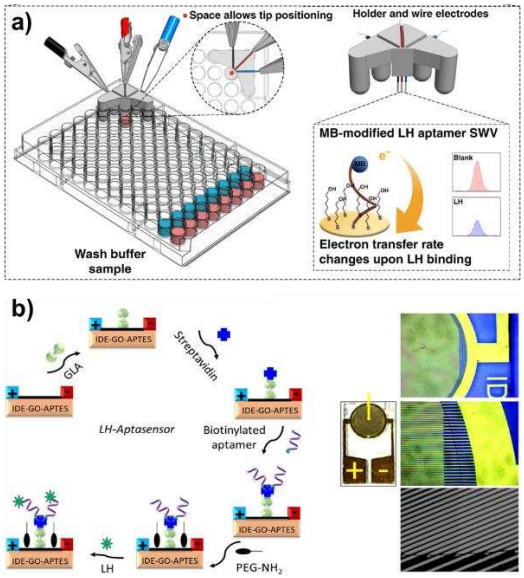


Fig. 7. Schematic representation of the aptamer-based sensing approaches for quantification of Luteinizing Hormone (LH). Reproduced with permission of **a)** (Liang et al., 2019), Open Access, Nature Communications, Creative Commons license; and, **b)** (Sun et al., 2021), Copyright (2021), Wiley-VCH.

3.3.2 Follicle-stimulating Hormone

The human FSH, also called follitropin, is a glycoprotein of approximately 207 amino acids (Padmanabhan et al., 2018) (**Fig. 1c**) secreted by the basophil cells in the anterior pituitary gland. It is a dimeric protein, consisting of an alpha and a beta subunit, of which the latter interacts with the FSH receptor (FSHR) (Ibrahim, 2013). It is primarily the pulsatile release of GnRH, from the medial basal hypothalamus, that causes the production and release of FSH from the gonadotropic cells of the anterior pituitary, represented in **Fig. 1b**. In females, FSH binds to granulosa cells in ovaries, stimulating the release of inhibin. It also stimulates the production and release of estrogen/E2, as a steroid hormone regulating FSH production and release (Ibrahim, 2013). The FSH plasma levels of healthy women in different stages of life and the ovarian cycle can be found in **Table 1.**

FSH is essential for proper reproductive development being crucial for sustaining ovarian follicles through the activation of over 100 different genes, all of which lead to multiple physical changes such as increased vascularization of the theca internal layer of cells near the basal lamina and formation of a fluid-filled antrum within the growing follicular cell (Dias et al., 2002). FSH effectively prepares the uterus for the implantation of a fertilized egg (Hunzicker-Dunn and Maizels, 2006). As levels of inhibin increase, FSH levels decrease and remain low until the end of the luteal phase, the latter stage of menstruation, in which a renewed, steady rise in FSH prepares more follicular cells for the next ovulatory cycle (Hunzicker-Dunn and Maizels, 2006).

As for the other aforementioned hormones, the conventional ways to test FSH are using immunoassays and expensive lab-bench methods. Despite the importance of this hormone in the control and management of the ovulatory and reproductive cycle, there is no aptamer suitable for the recognition of FSH. The only related publication on this topic is a work developed by Ibrahim *et al.* in 2013. The main focus and goal of the study were to develop an RNA aptamer (**Table 2**) that binds specifically to the human receptor of this hormone, herein named FSHR, *in vitro* by using a modified cell-SELEX procedure (Ibrahim, 2013). The FSHR belongs to a class of large molecules called G-protein coupled receptors which are embedded in the cell membrane. Conveniently, the study is based on the principle that many of the RNA sequences bind to the protein receptors on the cell surface. The idea behind the design was the generation of an aptamer to bind to FSHR and thereby will ultimately inhibit FSH from binding. The author hypothesized that this will stunt follicular development, thus acting as a form of oral contraception, most likely designed for males (Ibrahim, 2013). The interesting approach offers a new way of thinking for contraception for males (i.e., by controlling sperm production via regulation of FSH) rather than exposing women to continuous doses of hormones for the same purpose. The authors claimed that the novel approach would open the door to a new, safer, and possibly more cost-effective form of contraception that does not introduce extra hormones into the human body (Ibrahim, 2013). Ultimately, this approach could also be applied as a sterilization method for pets, which would reduce euthanasia rates in pet shelters (Spehar and Wolf, 2019). In any case, deeper studies must be performed to confirm the binding capabilities of the proposed RNA aptamer as well as the understanding of the mechanism interfering with the final FSH binding which will depend ultimately on the type of interaction between the aptamer and the FSHR (i.e., allosteric *versus* umbrella agonist/antagonist) as the authors claimed (Ibrahim, 2013). Therefore, this research line needs further intensive exploration from different perspectives, particularly in characterizing and enhancing the specific binding between the RNA aptamer (shown in **Table 2**) and FSHR, as well as designing new aptamers against the hormone FSH.

4. Future challenges: advantages and disadvantages of using aptamers for fertility monitoring

The accurate and fast measurement of reproductive hormones is urgently needed for several reasons: ***i)*** the crucial roles that the four key hormones play in our reproductive system in determining how fertile a woman can be, ***ii)*** their importance in terms of tracing the evolution of a patient suffering from a disease associated with a hormonal imbalance, and ***iii)*** since they can be considered and classified as emerging contaminants (as EDC), a growing concern due to their negative impacts on humans, aquatic life, and the environment have also been highlighted. Therefore, technologies for hormone quantification, that could be adapted for continuous sensing in the future (i.e., normal daily routine for individual use), would revolutionize the clinical care of patients with reproductive disorders (Liang et al., 2019).

In general, the well-established and routine clinical monitoring of hormones (e.g., by using immunoassays, LC-MS, or other lab-bench methods) to identify the normal or altered profiles is not feasible because it is extremely resource-intensive and time-consuming (Prante et al., 2020; Thomas et al., 2008). To overcome the limitations of such conventional methodologies, aptamer-based POC testing (POCT) has been proposed as an alternative. However, challenges remain (**Fig. 8**) to be tackled such as affordability, user-friendliness, and the ability to reach lower LODs aiming for non-invasive samples (e.g., saliva, in which the concentration range of the hormones is lower than in blood or urine). Importantly, data analysis is needed to properly study the correlation between different matrices (i.e., blood *versus* saliva levels). Microfluidic designs can offer certain advantages for this transition to POCT, also decreasing the required sample volume. Interest in the development of simple LFA formats using alternative bio-recognition elements such as aptamers, particularly for small molecule targets, has been increasing in recent years. However, constructing aptamer-based LFAs for small molecule targets, where only one aptamer can specifically bind one target at a time, is more challenging, requiring indirect signal transduction mechanisms to be engineered (Alsager et al., 2017).

Although aptamers have been suggested for numerous sensing applications, their translation into commercial applications is still in its infancy (Prante et al., 2020). This can be partially explained by the lack of a thorough characterization and validation of aptamer-target interactions as described by Zhao *et al.* (Bottari et al., 2020; Zhao et al., 2021). Hence, the use of complementary analytical techniques (CAT in **Fig. 8**), with extensive negative and positive controls, is proposed for characterizing the binding interactions and mechanisms. The use of CAT facilitates an in-depth understanding of affinity, selectivity, mechanism, and

structure, tackling the specificity conundrum of several published aptamers (Bottari et al., 2020; Cai et al., 2018). Interestingly, most concerns revolve around small molecule-binding aptamers. One possible explanation is their rather limited number of functional groups, which are important for specific target recognition and binding. For instance, it was shown that hydrogen bonds are extremely important in the theophylline-binding aptamer for target recognition and binding (Zimmermann et al., 1997). Consequently, fewer functional groups lead to decreased specificity and affinity. This poses a problem for the development of new aptamers for small molecules since the target analytes are usually monitored in the nM range (regarding health markers) and require high specificity (Prante et al., 2020). Recently, Miller *et al.* reported that many aptamers are truncated without a clear justification and/or characterization, leading to a propagation of mutated sequences in the literature compared to the original aptamer (Miller et al., 2022). The use of CATs for studying truncated aptamers could help in understanding their binding interactions and avoid issues in aptamer research.

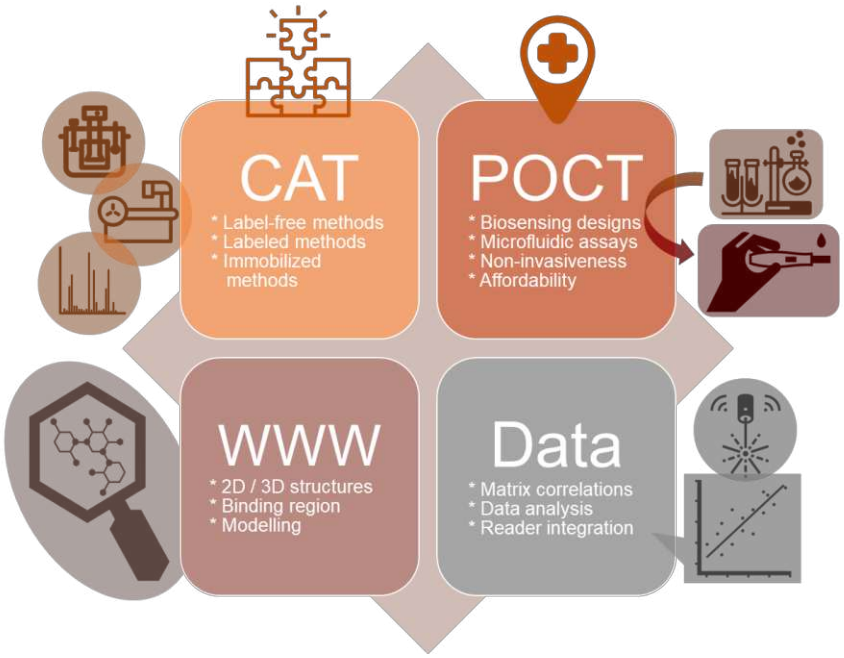


Fig. 8. Diagram of the current challenges and future improvements in the development of an accurate user-friendly device for fertility monitoring. Complementary Analytical Techniques (CAT) to help in the understanding of the interaction between the aptamer and the target; point-of-care Testing (POCT) towards the decentralization of such platform being non-invasive, affordable, and accessible; utilization of the World Wide Web (WWW) online tools for predicting and modeling of the structure and binding site of the aptamer; and Data analysis for data processing, correlation of the ranges of interest in different matrices and samples, and the reader integration into a portable device.

Modeling-based studies and modeling tools are essential to map the binding site of the aptamer promoting a better understanding of the interaction, which can then be used to modify the aptamer to increase affinity and specificity (Alhadrami et al., 2017) (**Fig. 8**). These types of studies can bring light to the recognition of the specific binding site and their profound characterization such as the example provided by Eisold and Labudde (Eisold and Labudde,

2018). In that specific study, the authors offered a detailed analysis of the interactions between an aptamer (E2-35) and E2 using molecular dynamic simulations, which ultimately contributed to a better understanding of the behavior of target binding with aptamer structures in aqueous solution. Mfold web server is also highlighted here, which describes several closely related software applications available on the World Wide Web (WWW) for the prediction of the structure of nucleic acid sequences (Zuker, 2003). Another platform that can be used for estimating the two-dimensional aptamer structure is NUPACK, which was employed for generating the structures in **Table 2** ("http://www.nupack.org/," 2022; Yu et al., 2021). These tools are computational methods that provide the structure of the aptamer, but also other interesting parameters such as the free energy of the aptamers complexed with complementary oligonucleotides. The structures supplied by computational methods need, however, further experimental characterization to verify their accuracy (Miller et al., 2022). Three-dimensional structures are more challenging but not impossible such as the example offered by Eisold and Labudde.

All these implementations and advances together with the use of algorithms and adequate data treatment, as well as the integration of the reader in a portable platform, could ultimately favor the development of truly efficient devices able to accurately monitor the concentration profiles of the four hormones responsible for ovulation in females.

5. Conclusions

Despite the remaining challenges related to the use of aptamers in biosensing approaches still to be tackled, the scientific community is undertaking great efforts to release thoroughly characterized aptasensing approaches for accurate and sensitive fertility monitoring. This review summarizes and discusses all aptasensing-based publications on this field emphasizing the necessity of a better understanding of the binding interactions and mechanisms. From our point of view, there is still a lack of complete studies in which this profound understanding is fulfilled while providing a robust platform. From an analytical perspective, it is also challenging to find the best option and select the approach, method, and/or design for sensing purposes since the optimal option could be defined and be influenced by several factors such as the sample type (i.e., blood, serum, saliva, etc.), final application (early diagnosis, prognosis, etc.), and limit of detection (quantitative versus qualitative), among others. Moreover, clinically relevant ranges of hormones are not only dependent on the selected matrix since the levels of the same hormone in different types of samples are not well-established and correlated yet in most cases.

Therefore, the integration of well-characterized, validated, and specific aptamers with high affinity into sensing platforms, either optical or electrochemical, will enable reaching clinically relevant levels of all fertility-related hormones. Although all different sensing approaches shown within this review have advantages and disadvantages, thinking about further improvements to achieve an ideal and reliable platform could be based on a mixture of the best features of both optical and electrochemical readouts. Hence, the most promising and time-to-come aptasensing approaches might be anchored on photoelectrochemistry or similar combinations of those analytical techniques. Bearing this in mind, forthcoming works could offer, in the near future, user-friendly, non-invasive, and reliable tools for facilitating fertility monitoring, disease tracing, and personalized medicine. Overall, these POC devices will provide the end-user the opportunity to monitor key (bio)chemical information of our body, from home and in an intimate way, for enhanced control of the fertility window.

Author Contributions

Rocío Cánovas: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Elise Daems**: Writing – review & editing. **Amelia R. Langley**: Writing – review & editing. **Karolien De Wael**: Supervision, Funding acquisition, Writing – review & editing.

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Conflict of Interest statement

The authors declare no conflict of interest.

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