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# Aptasensing luteinizing hormone to determine gynecological endocrine complications on graphene oxide layered sensor

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**Abstract**

Luteinizing hormone (LH)/lutropin is an interstitial cell-stimulating hormone playing a predominant role in the reproductive system, and highly correlated with the infertility treatment in both men and women. This research was concentrated to quantify LH level by using interdigitated electrode sensor. To improve the electric current flow, sensing electrode was modified with graphene oxide (GO) and the aptamer probe was attached on GO through biotin-streptavidin linker. Current responses were measured with aptamer–LH interaction at the target concentrations between 7.5 nM and 1 $\mu$M and the detection limit of LH was calculated as 60 nM with the determination coefficient ($R^2$) value, 0.9229 [$y = 1.296x - 2.8435$] on a linear range from 30 nM to 1 $\mu$M. Further, biofouling effect on sensing electrode surface was analyzed with complementary aptamer sequence, control proteins (albumin and globulin). The above GO-aptamer-modified interdigitated electrode sensor helps to quantify LH level and diagnose gynecological endocrinology-related complications.

**KEYWORDS**
aptamer, biomarker, biosensor, endocrinology, graphene oxide

## 1 | INTRODUCTION

Gynecologicalendocrinology involves menopause, infertility, and other issues with the reproductive hormones. It is highly mandatory to monitor certain hormones in the reproductive system to treat and improve the gynecological-associated problems such as infertility.

**Abbreviations:** LH, Luteinizing hormone; GO, Graphene oxide; $\mu$M, Micromolar; nM, nanomolar; mL, milliliter; PCOS, polycystic ovary syndrome; APTES, 3-(Aminopropyl)-tryethoxysilane; GLA, Glutaraldehyde; IDE, Interdigitated electrode; KOH, Potassium hydroxide; PBS, Phosphate buffered saline; PEG-$NH_2$, Polyethylene glycol-aminated; SEM, Scanning Electron Microscope; EDX, Energy Dispersive X-Ray Analysis.

Reproductive endocrinology also regulates the sexual function, development, and reproduction.[1,2] Reproductive endocrinology disorder occurs when there are abnormal changes in hypothalamus–pituitary gonadal axis, and leads to various gynecological-related problems, which include virilization, infertility, and hirsutism.[3–5] Luteinizing hormone (LH) is an interstitial cell-stimulating hormone, which helps reproductive system, in particular for men's testes and women's ovaries.[6] LH is made by pituitary gland; it is a critical enzyme for synthesizing the mature oocytes and for the downstream regulation of synthesizing sex steroid hormone. The secretion of LH is highly correlated with hypothalamic dysfunction, resulting in various reproductive disorders, such as hypothalamic amenorrhea and PCOS (polycystic ovary syndrome). Further, the level of LH helps to analyze the infertility and the problem in the pituitary gland. The major clinical use for exogenous LH administration is highly linked to the infertility treatment in both women and men. So that identifying and quantifying LH is mandatory to diagnose the fertility and gynecological endocrine-related problems. Quantifying the LH level is still challenging due to the inherent biological variation, impact of physiological factors on hormone secretion, and LH centration profile is cost restrictive. Developing a cheaper and efficient quantifying method for LH is mandatory with medical applications. This research work introduced a new graphene material-modified interdigitated electrode surface for developing a sensitive LH biosensor.

Graphene oxide (GO) is the promising material to be utilized in biosensors due to its large surface area, unique dispersion in medium, and biocompatibility.[7] The change in the electrical conductivity resulting from the molecular adsorption on graphene surface is the basic working principle with graphene-based sensor. The graphene-based material provides a microenvironment for analyzing the biological activity of the biomolecules, and promotes the electron transfer between the immobilized biomolecule and the electrode matrix.[8] The potential characteristics of GO, such as mechanical strength, electrical conductivity, and biomolecular adsorption, make it suitable for various downstream applications.[9,10] In the engineering aspects, GO has been considered in optoelectronics, energy storage media, supercapacitors, polymer composite, solar cells, batteries, and as catalysts.[11] Current applications of GO also include preparations of aerogel, fiber and composites, bioimaging, tissue engineering, drug carrier, anticancer agent, and recently with stem cell technology. More importantly, the surface groups (hydroxyl, ketone, carboxyl, and epoxy) on GO are highly suitable for biomolecular immobilization.[12,13] As biosensors highly rely on the surface chemical functionalization, especially with probe immobilization and alignment, GO is the right material to form sensing surface epilayers.[14,15] Therefore, the preparation of graphene-based biosensors is another important point of application with the immobilized enzyme/protein on the surface. Studies have shown graphene as the electrode material for $H_2O_2$ sensing with a linear response range of 1.2–15.25 $\mu$M, with a detection limit of 0.2 $\mu$M.[16] Chemical vapor deposition, epitaxial growth, mechanical cleavage, and chemical deoxygenation of graphene are the common methods in synthesizing graphene for biosensors, but among these approaches deoxygenation can be done at low cost and with high quality and quantity. In addition, GO can be used for vitro biosensing of DNA, ion and biomolecular detection and GO-based fluorescent biosensor has been utilized in the detection of small biologically active molecules and proteins.[17–19]

This study uses aptamer as the probe molecule and highly amenable to the properties of GO. Any desired aptamer can be modified at their end(s) chemically, which creates a perfect partner to GO. Apart from these, aptamer has originally stem-loop folding pattern that can be predicted with a possible secondary structure. The presence of loop region(s) makes aptamer interact with the target at high affinity.[20,21] Further, the affinity modes as aptamer-induced target binding, target-induced aptamer binding, and co-induced interaction are the backbone principles with aptamer.[22] Several successes with aptamer-based high-performance sensors (aptasensors) have been demonstrated due to the right orientation of aptamer during the immobilization process.[23] The success of aptasensors is also due to the smaller aptamer size to accommodate more targets on the sensing surface, which elevates the sensitivity level of the sensors.[24,25] Along with GO, aptamer has proved to be a good partner for the sensing applications especially in the field of medical diagnosis.[26]

**Highlights**

- This research was concentrated to quantify Luteinizing hormone level on interdigitated electrode sensor. Sensing electrode was modified by graphene oxide and with aptamer as probe the detection limit was calculated as 60 nM at the determination co-efficient (R2), 0.9229 [$y = 1.296x - 2.8435$] on a linear range from 30 nM until 1 $\mu$M. Biofouling effect on sensing electrode surface was analysed with complementary aptamer sequence, control proteins (BSA, and globulin).

Scientists are focusing on the development of GO by using greener technologies, where they use a cleaner reductant in the deoxygenation process. Several green reductants such as bacteria, commercial biomolecules, and phytoextracts have been used in the deoxygenation of GO.[27] Among all these green reductants, phytoextracts are the most promising reductants as the plant can be easily obtained and is less expensive. Making use of medicinal plant is a better solution to overcome the poisonous GO from chemical deoxygenation process because phytoextract is less toxic or nontoxic compared to chemical and synthetic drugs. In addition, using phytoextract makes the deoxygenation process cost-effective, ecofriendly, and most importantly cleaner. The important compounds in phytoextracts are hydroxyl and carbonyl groups, thus the capability to serve as capping and reducing agents in the reduction process.[28] The use of different phytoextracts as reducing agent for the GO reduction is intensively studied, as phytoextracts consist of polyphenolic and flavonoid compounds that can be easily oxidized and have shown excellent antioxidant properties.[29] In this work, GO was synthesized by using *Phyllanthus niruri* leave extract as a reduction agent, and the synthesized GO was used to capture the aptamer for quantifying the level of LH.

## 2 | MATERIALS AND METHODOLOGY

### 2.1 | Reagents

3-(Aminopropyl)-tryethoxysilane (APTES), glutaraldehyde (GLA), LH, polyethylene glycol-$NH_2$ (PEG-$NH_2$), sulfuric acid, graphite powder, and serum albumin were purchased from Sigma Aldrich (MO, USA). Interdigitated electrode (IDE) was obtained commercially from Metrohm (China) and the adopted aptamer sequence (5' forward primer-TATGGTATGCTGTGTGG TATGGGGTGGCGTGCTCT-reverse primer 3') was synthesized commercially.[30]

### 2.2 | Preparation of *P. niruri* leave extract and graphene oxide

One gram of plant *P. niruri* fresh and healthy leaves were collected and transferred into a tube containing 40 ml of distilled water and soaked overnight. The next day, the solution was heated at 60°C for 20 min, and then centrifuged at the speed of 12,000 rpm for 5 min at 20°C. The solution is then filtered again by using single-use filter unit. The leave extract was stored in the refrigerator for further use.

One gram of graphite powder was suspended in 25 ml of sulfuric acid using an ice bath with stirring continuously. Then, 3 g of potassium permanganate was added gradually over a period of 30 min with constant stirring in a round bottom flask and at <14°C. Then the ice bath was removed and stirred until it appears pasty brownish and continuously stirring for ~2 h. After that, add 50 ml of water was added, the reaction temperature was increased to 96°C, and the color changes to brown. Further, 100 ml of distilled water was added to weaken the solution and then 5 ml of hydrogen peroxide added and the reaction was stopped. The prepared GO was separated by centrifugation at 12,000 rpm for 5 min at 20°C and rinsed with distilled water. Final product of GO was air-dried in hot-air oven and the absorbance of the resulting products analyzed by scanning electron microscopy.

### 2.3 | GO-modified LH sensor preparation

GO was attached on the electrode surface through amine chemical modification. For that the surface was immersed in 1% of KOH for 15 min and then GO-mixed APTES (1 g of GO in 1% diluted APTES) was added on the surface and kept for 2 h. The unreacted GO was eliminated by washing the surface by diluted ethanol. Further, 2% of GLA was added and after removing the excess aptamer on the surface by PBS, the uncovered surface was covered with 1 mg/ml of diluted PEG-$NH_2$. This LH–aptamer-modified GO surface was used to identify LH level.

### 2.4 | LH identification and limit of detection: Probe–target interaction

LH level was quantified on GO-anti-LH aptamer-modified IDE sensor. LH concentrations from 7.5 nM to 1 $\mu$M were diluted in 10 mM PBS (pH 7.4), interacted independently on aptamer-attached surfaces, and then current changes were recorded after removing the unbound LH by PBS. The differences in voltage were calculated for each LH concentration and then plotted in an Excel sheet to identify the limit of LH detection.

### 2.5 | Biofouling on GO sensor: Specificity

Biofouling of GO-modified IDE electrode was analyzed with three different control molecules, namely complementary aptamer sequence and control proteins (albumin and globulin). In addition, to identify the specific recognition of LH, it was spiked in 1:100 dilution of human serum and then the interactive analysis was conducted. The current voltage measurements for the above experi-

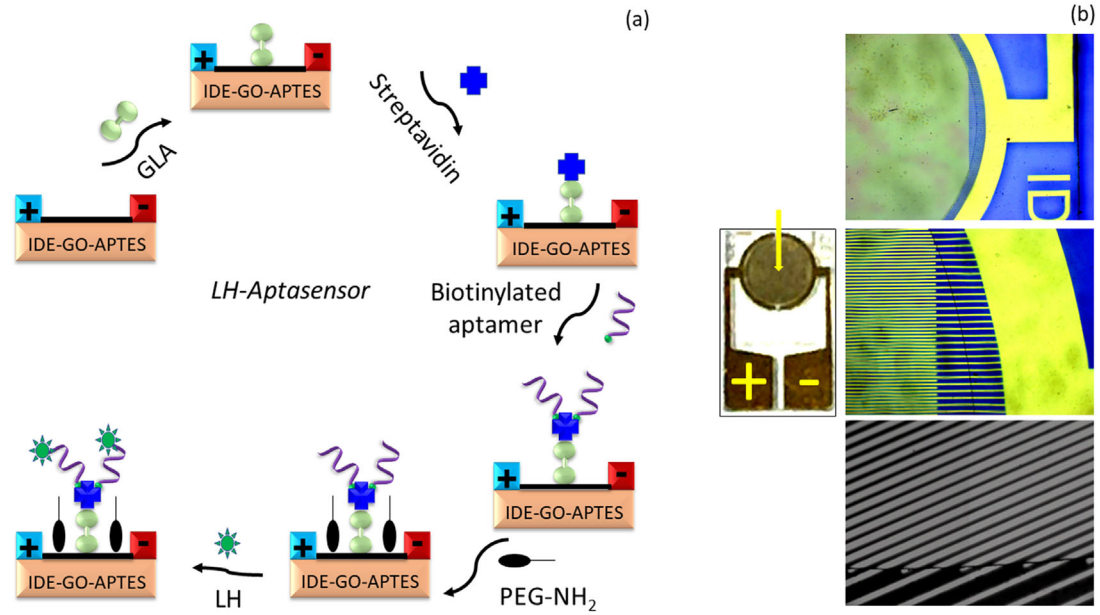


**FIGURE 1** (A) Schematic representation of LH detection on GO-aptamer-modified IDE electrode surface. Biotin-streptavidin linker was used to attach the aptamer on GO-modified electrode surface. GLA was used to make a link between APTES and streptavidin. (B) Device and surface appearance. Commercial device and high-power microscope were used to capture the electrode surface. Arrow indicates the sampling area and "+" and "–" are points of current probing

ments were recorded and compared with specific detection of LH. All experiments were performed in wet condition and washings steps followed between each surface modification. Keithley ammeter was used for all measurements at triplicates from 0 to 2 V, with the intervals of 0.1 V for 2 min.

## 3 | RESULTS AND DISCUSSION

Figure 1A shows the schematic representation of LH detection on GO–aptamer-modified IDE sensor. Figure 1B displays photograph of IDE sensor and the surface electrode structures captured by high-power microscope. Biotin-streptavidin linker was used to attach the aptamer on GO-modified electrode surface. Initially, GO was modified on the electrode through the APTES linker. GO-dispersed APTES was attached on the electrode surface through the interaction of hydroxyl and the amine group from the APTES. Further GLA was used to make a link between APTES and streptavidin, then biotinylated aptamer was attached. PEG-$NH_2$ was dropped to cover the excess GLA surface to avoid nonspecific binding of LH on GLA. In this research, anti-LH aptamer was used as the probe to identify and quantify LH. Aptamer is artificial antibody, more sensitive and selective with their target molecule, which can identify specifically the target at lower level.[31–33] Apart from that, in most cases, small region of loop structure in the aptamer involves binding with target and helps to differentiate the closely related molecules.[34] Lakshmipriya et al. found that antibodies cannot differentiate the types of influenza virus, while aptamers can differentiate even subtypes, which helps to identify the target molecule with more specificity.[35] The current research has utilized LH-specific aptamer to identify LH level at its lower abundance for diagnosing the gynecological endocrine-related problems.

### 3.1 | Characterization of synthesized GO

The morphology and elements in the synthesized GO were analyzed by scanning electron microscope (SEM, Hitachi, Japan). Figure 2A,B shows SEM images of GO at the scales of 5 and 1 $\mu$m. The synthesized GO was distributed uniformly with the flake-like structure. The EDX analysis confirms the presence of carbon (65.49%) and oxygen (22.24%) (Figure 2C).

### 3.2 | Attachment of aptamer on GO surface

Aptamer was attached on the GO surface through biotin-streptavidin linker. The current response for this process was recorded to confirm the binding of aptamer on the sensing electrode surface. As shown in Figure 3A, IDE-GO-APTES-treated surface shows the current level

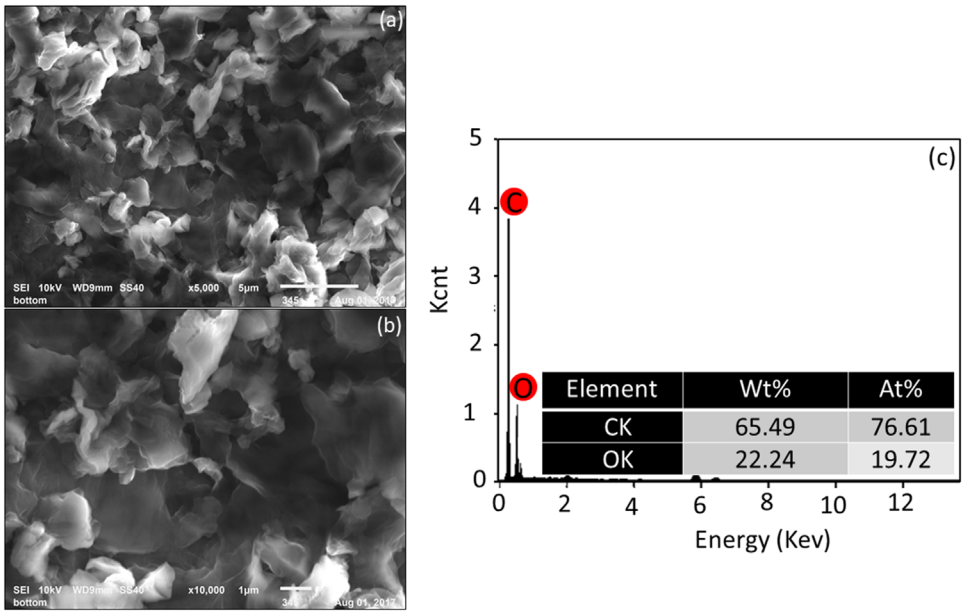


**FIGURE 2** (A) Characterization of synthesized GO. GO was distributed uniformly. SEM image of GO at a scale of 5 $\mu$m. (B) SEM image of GO at a scale of 1 $\mu$m. (C) EDX result of GO. Higher percentages of carbon and oxygen were found

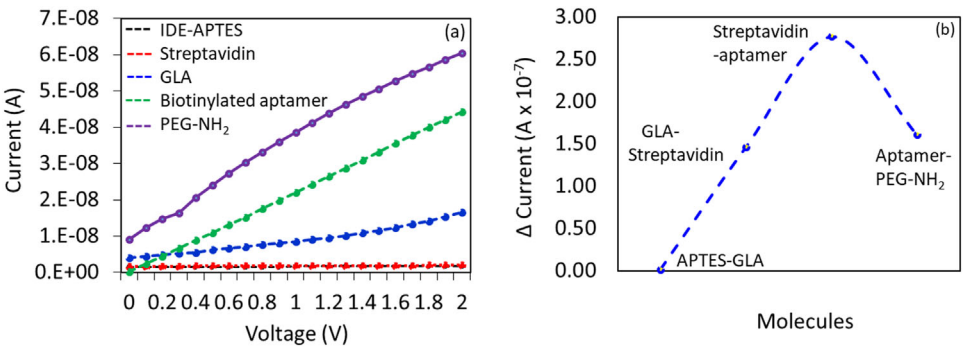


**FIGURE 3** Process of aptamer attachment on GO surface. (A) Current responses of each biomolecular immobilization. Clear increment of current was noted after each biomolecular attachment. (B) Current differences with each biomolecular immobilization. Aptamer attachment shows the highest changes in current difference

as 1.76E-09 A, after drop GLA current level was changed to 1.95E-09 A, which confirms the attachment of GLA to the immobilized APTES surface. Further, when streptavidin was added, the current was elevated to 1.66E-08 A, this increment shows the immobilization of streptavidin on GLA surface. After that, biotinylated LH–aptamer was dropped, then current level was dramatically increased to 4.43E-08 A. It has been proved that binding sites on streptavidin can attract four biotin molecules, which helps to exhibit higher number of biotinylated aptamers on the sensing electrode surface.[36] Higher number of aptamer-immobilized surfaces attract higher number of LH, which improves the detection limit. As a big change of current response was noticed after adding biotinylated aptamer, it confirms the higher number of aptamers on streptavidin-modified electrode surface (Figure 3B). Finally, the blocking agent, PEG-NH2, was dropped to cover the remaining GLA surface. PEG and their derivatives are widely utilized to reduce the biofouling effect on the sensing surface[37] and in the current study used as the blocking agent to mask the areas free from probe attachment. Due to the unique interaction between PEG and water molecules, it results in the formation of hydrated layer and prevents the biofouling. In addition, with the smaller size and immobilization at right orientation, PEG accelerated the genuine interaction. In addition, PEG-based blocking not only helps to reduce the signal-to-noise ratio, it also gives the protection to the immobilized biomolecule by providing a proper orientation.[23,37] This aptamer-attached surface was used as the identification electrode platform to quantify LH level.

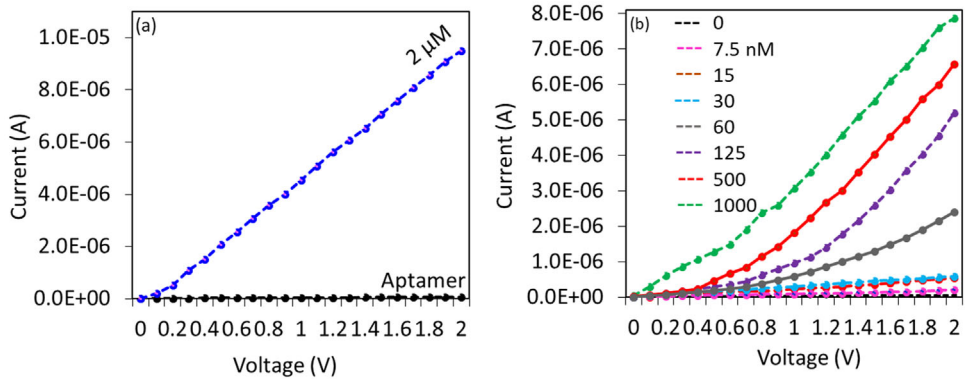


**FIGURE 4** Determination and quantification of LH. (A) At higher concentration, 2 $\mu$M was dropped on aptamer-immobilized surface and a clear current response was noted. (B) Dose-dependent analysis. LH was diluted from 7.5 nM to 1 $\mu$M and dropped independently on aptamer surfaces to identify the interaction of LH. With each concentration/interaction, changes in current were noted

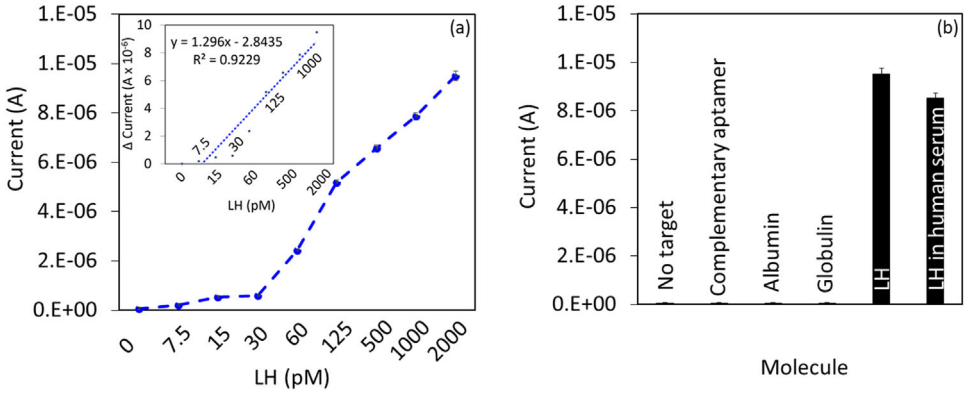


**FIGURE 5** (A) Current response of LH concentrations from 7.5 nM to 2 $\mu$M. With the increment in the concentration of LH, current volt changes were increased gradually. Inset shows the difference of volt before and after interaction of LH and aptamer. The detection limit of LH was identified as 60 nM with $R^2$ value 0.9229. (B) Biofouling effect on GO-aptamer electrode surface. Three types of molecules, namely, complementary aptamer sequence, albumin, and globulin were used to check the biofouling. There were no significant current responses recorded, indicating the specific interaction of LH with aptamer. Selective experiment conducted with LH-spiked serum, and there is a clear difference of current noted upon interaction of LH with aptamer. Error bars are with averaged values

## 3.3 | Determination and quantification of LH

Different concentrations of LH were identified individually on LH–aptamer-modified GO surface. At first higher concentration (2 $\mu$M) of LH was dropped on aptamer-immobilized surface and the current response was noted. As displayed in Figure 4A, current was changed from 6.04E-08 to 9.51E-06 A, which confirms the aptamer-modified sensing electrode to recognize LH. Further, LH was diluted from 7.5 nM to 1 $\mu$M and dropped on aptamer-attached surfaces to identify the interaction of LH with its aptamer. As shown in Figure 4B, 7.5 nM (6.04E-08–2.06E-07 A) shows slight change with current difference, and then with increasing concentrations of LH to 15, 30, 60, 125, 500, and 1000 nM, current levels were improved to 5.39E-07, 5.89E-07, 2.41E-06, 5.19E-06, 6.58E-06, 7.87E-06, and 9.51E-06 A, respectively. Clearly it was noted that with increasing concentration of LH, current volt also increased gradually (Figure 5A). The differences in volt before and after each interaction of LH and aptamer was calculated and then plotted in an Excel sheet. The detection limit of LH was identified as 60 nM with the determination coefficient ($R^2$ value) of 0.9229 on the linear range of 30–1000 nM (Figure 5A; inset).

## 3.4 | Biofouling of control molecules on GO-aptamer-modified electrode surface

It is mandatory to identify the biofouling on the probe-modified sensing electrode surface to confirm the specific detection of LH. In this research work, PEG was used as the blocking agent to control the binding of other biomolecules

on the GLA surfaces. Three types of molecules, namely, complementary aptamer sequence, albumin, and globulin were used to check the biofouling effect of probe-modified sensing electrode surface. When complementary aptamer was used instead of specific aptamer, no significant current was noted, confirming the failure of binding of LH with complementary aptamer. Further, when albumin and globulin were dropped on aptamer-modified surfaces instead of LH, there was no current response recorded (Figure 5B). Globulin (2.6–3.5 g/dl) and albumin (3.5–5.0 g/dl) are the major and predominant proteins in the blood serum with the molecular sizes of ~150 and 66.5 kDa, respectively.[38] Present study is embarked to demonstrate the genuine interaction between aptamer and LH in the presence of these proteins, which supports the condition with the real sample analysis. These overall results confirm that our aptamer-modified sensing electrode surface did not show any biofouling effect and it only allows the specific interaction of LH with aptamer. In addition, to identify the selective recognition of LH, it was spiked in 1:100 dilution of human serum and then the experiment for LH and aptamer interaction was conducted. As shown in figure, there was a clear difference of current noted upon interaction of LH with aptamer, indicating the identification of LH in the real-life sample without interference of other biomolecules. The primary success of the current volt aptasensor is shown here due to the transducing mechanism with sensing by dielectrodes. This dielectrode is operating by the dipole moment between two electrodes during the molecular/biomolecular interaction or attachment. In other words, there is an unequal sharing of electrons between two electrodes, and the calculation is made by measuring the net molecular polarity forming at either ends of molecular dipole.[39,40]

## 4 | CONCLUSION

A highly sensitive LH biosensor was developed on GO complexed interdigitated electrode surface to identify the gynecological endocrinology-related issues. Aptamer was used as the capture probe and attached on GO surface through the amine-aldehyde chemical linkers. LH was identified on these aptamer-modified surfaces, and the current responses were gradually increased upon increasing the LH concentrations. The limit of LH detection was identified at 60 nM and the control molecules, complementary aptamer sequence, albumin, and globulin fail to show the current responses, indicating the specific LH detection. This research work demonstrated to quantify LH level, which can help during the treatment of infertility, further can be followed for detecting other clinical biomarkers. Even though, the current sensor demonstrated high performance well, when changing the surface with another material, it needs further optimization. Another limitation is with the gap size between fingers to be microscale and when reduced to the nanoscale level, the sensor gets short-circuit with the output signal.

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