Quantification of cortisol for the medical diagnosis of multiple pregnancy-related diseases

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Abstract

Cortisol is a stress hormone released from the adrenal glands and is responsible for both hyperglycemia and hypertension during pregnancy. These factors make it mandatory to detect the levels of cortisol during pregnancy to identify and treat hypoglycemia and hypertension. In this study, cortisol levels were quantified with an aptamer-conjugated gold nanorod using an electrochemical interdigitated electrode sensor. The surface uniformity was analyzed by high-power microscopy and 3D-nanoprofiler imaging. The detection limit was determined to be 0.01 ng/mL, and a linear regression indicated that the sensitivity range was in the range of 0.01–0.1 ng/mL, based on a 3σ calculation. Moreover, the specificity of the aptamer was determined by a binding analysis against norepinephrine and progesterone, and it was clearly found that the aptamer specifically recognizes only cortisol. Further, the presence of cortisol was detected in the serum in a dose-dependent manner. This method is useful to detect and correlate multiple pregnancy-related diseases by quantifying the levels of cortisol.

Keywords Hyperglycemia · Gestational hypertension · Aptamer · Gold nanorod · Interdigitated electrode · Human serum

Introduction

Hypoglycemia and hypertension are conditions that may occur during pregnancy. Hypoglycemia is characterized by a lower than normal level of glucose, less than 60 mg/dL, due to changes in the regulation and metabolism of glucose. In women with type I diabetes and a higher risk of hypoglycemia, there is a strong relationship between fetal and maternal glucose levels during both late and early gestation. The level of blood glucose plays an important role in providing energy to the fetus during pregnancy. A constant level of glucose is needed to maintain good health in the mother and fetus, whereas high and low levels cause various problems during and after delivery. In general, gestational diabetes has been

observed between weeks 24 and 28; consequently, the fetus may experience hyperglycemia (high glucose levels) prior to the testing frame. Thus, it is mandatory to identify gestational diabetes earlier, at least during weeks 18–20. Maternal hypoglycemia affects both the mother and conceptus and needs to be continuously monitored during pregnancy.

Similarly, hypertension is another major problem that occurs widely during pregnancy. Gestational hypertension or pregnancy-induced hypertension is a complication in 10% of pregnancies and causes poor perinatal outcomes and illnesses, such as elevated arterial blood pressure, eclampsia and pre-eclampsia, during pregnancy (Kelder et al. 2012; Poprawski et al. 2012). In addition, these changes affect other parts of the body, such as the heart and kidney, and may cause early delivery. In general, the above issues appear during the second half of pregnancy, and identifying hypertension and hypoglycemia by means of a suitable biomarker is necessary for a healthy pregnancy (Sibai 2003; Magee et al. 2014).

Cortisol is a stress hormone secreted by the adrenal gland, and an elevated level of cortisol is found in the body during high-stress conditions (Dedovic et al. 2009; Matousek et al. 2010). Cortisol is released into the bloodstream directly by the adrenal glands, and it has been proven that serum cortisol plays a pivotal role in the pathophysiology of gestational

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hypertension and hypoglycemia (Vianna et al. 2011; Kosicka et al. 2018). 11 β -Hydroxysteroid dehydrogenase type 2 (11 β -HSD2) is an enzyme produced in the renal tubules that converts cortisol into inactive cortisone, allowing the mineralocorticoid receptor to be aldosterone-selective. Diminished function of this enzyme is caused by various mutations in the HSD11B2 gene, which encodes 11 β -HSD2, and this diminished function is responsible for an inherited form of hypertension known as apparent mineralocorticoid excess (AME). This syndrome is characterized by excessive F (cortisol), which interacts with the mineralocorticoid receptor, results in sodium and water retention and eventually causes hypervolemic hypertension (McCalla et al. 1998; Morineau et al. 2006; Ferrari 2010; Kosicka et al. 2015). Moreover, under stressful conditions, cortisol provides the body with glucose by tapping into protein stores via gluconeogenesis in the liver. This energy can help an individual fight or flee a stressor. However, elevated cortisol levels over the long term produce glucose consistently, leading to increased blood sugar levels (Andrews et al. 2002). Thus, quantifying the level of cortisol is necessary for identifying the root causes of both hypoglycemia and hypertension.

Preparing an easier sensitive detection system for cortisol is appealing for the generation of a dual-detection system for hypoglycemia and hypertension. A few studies have reported the use of nanorod-antibody conjugates, and our previous report described the use of nanorod-aptamer conjugates in a colorimetric assay. In the current study, we would like to expand this research to a different platform to obtain more insights and focus on a new aspect. Thus, we used a gold nanorod-conjugated aptamer as a probe to detect low levels of cortisol with the assistance of an electrochemical Interdigitated electrode (IDE) sensor. The aptamer is DNA or RNA, known as an artificial antibody, and was generated from a random library of molecules by the 'SELEX' (systematic evaluation of ligands by exponential enrichment) method in three simple steps, namely, binding, separation and amplification (Shangguan et al. 2008; Lakshmipriya et al. 2013b). Application of aptamers is widespread in various fields, such as medical diagnosis, environmental science, and drug delivery. Since aptamers are highly selective and sensitive to their target molecule, aptamer applications are most often focused in the field of medical diagnosis (Kim et al. 2010; Gopinath et al. 2012). Different aptamers have selectivity against a wide range of targets for the detection of cancer, viral and bacterial infections and metal ions (Gopinath et al. 2011; Wu et al. 2012; Ma et al. 2016). Various sensors utilize aptamers to quantify the level of cortisol (Sanghavi et al. 2016; Fernandez et al. 2017; Dalirirad and Steckl 2019).

In this work, we desired to use an aptamer selective toward cortisol for detection and quantification on an IDE sensing surface. This is a dielectric sensing system that has

been confirmed to be reliable, and the sensing electrode has a finger-like pattern with uniform gaps. The gap areas are minimal and have an optimized size to avoid short-circuits during measurements. Sample volumes on the order of microliters are needed for proper current measurements. Upon surface chemical modifications and biomolecular assembly, changes in the electric flow will occur. Upon molecular attachment, a dipole moment will occur between the electrodes and will cause variations in the current and voltage, reflecting molecular attachment onto the surface. Ideally, the surface can be modified physically by the nano-material to increase the surface area. Ultimately, more molecules can be accommodated on the surface of the proposed IDE sensing system and measured electrically, which reflects the dipole moment between the electrodes. In this context, gold nanorods were used to enhance the sensitivity. Among a wide range of metals, gold is preferable in the field of biosensors due to its appealing properties (Pavlov et al. 2004; Martin et al. 2014). Various sensing techniques, such as surface plasmon resonance, waveguide-mode sensing, colorimetry, and Raman spectroscopy, were used with a gold surface to improve the detection (Horiguchi et al. 2013; Lakshmipriya et al. 2014). Herein, we conjugated the aptamer with gold nanorods to improve the detection of cortisol with IDE sensors.

Materials and methods

Reagents and biomolecules

A cortisol aptamer, 5'-thiol-C6-GGAATGGATCCACATCCATGGATGGGCAATGCGGG GTGGAGAATGGT TGCCGCACTTCGGCTTCACTGCAGACTTGACGAAGC TT-3', was synthesized by a local oligonucleotide supplier. The hormones cortisol and progesterone were obtained from Adooq Biosciences (USA). Norepinephrine was purchased from Abcam (USA). Ethanolamine, 3-aminopropyltriethoxysilane (APTES), phosphate-buffered saline (PBS; pH 7.4) and human serum were obtained from Sigma Aldrich. Gold nanorods (GNRs) were procured from Nanocs, USA. A possible folding pattern of the aptamer with secondary structures was predicted by mfold online software (<https://unafold.rna.albany.edu/?q=mfold>).

Fabrication of Interdigitated electrodes (IDEs)

A silver IDE electrode was deposited on the silicon wafer sample <100> using the traditional wet-etching method. Initially, the silicon wafer was cleaned by standard cleaning solutions, and an aluminum IDE electrode was deposited onto the silicon wafer by the traditional wet-etching method. Then, a positive photoresist was deposited on the surface of

the silicon wafer, followed by thermal oxidation. Deposition of aluminum was performed by photolithography involving three steps: step 1, 1200 rpm for 10 s; step 2, 3500 rpm for 20 s; step 3, 500 rpm for 10 s. Then, the sensing surface was exposed to UV (ultraviolet) light to transfer the IDE pattern onto the sample surface. After that, the RD-6 developer was used for 15 s to carry out the developmental process. Photoresist deposition was performed to eliminate the unexposed regions. The developed sample was baked at 100 °C to remove unnecessary moisture and improve the adhesion between the SiO₂ layer and the aluminum. Finally, by applying an aluminum etchant for 23 s, the unexposed area was removed and cleaned by acetone. Using this basic preparation method, further modification via aptamer-GNR immobilization was performed by means of a chemical linker. High-power microscopy and 3D nanopprofilers were used to analyze the surface of the IDE. Images were captured using the associated system at 50× magnification.

GNR–aptamer conjugation

GNR–aptamer conjugation was carried out through a thiol-linker with a six-carbon spacer. To form the proper aptamer secondary structure, the aptamer was heated at 70 °C for 1 min and then cooled to room temperature. Upon cooling, the aptamer formed a stem-loop pattern to interact with the target. Then, 1 μM aptamer was mixed with 10 μl of as-received GNR (one optical density) and kept at room temperature (RT) for 30 min. After that, the unbound aptamers were separated by centrifugation at a speed of 10,000×g for 10 min. The supernatant containing the unbound aptamer was discarded. Then, the pellet was washed five times with distilled water and centrifuged as in the above steps. Finally, the obtained gold nanorod-aptamer conjugates in the pellet were kept at 4 °C for further use. The final conjugated sample was maintained under wet conditions to retain the aptamer secondary structure.

GNR–aptamer probe immobilization on an IDE surface

To prepare the probe-modified IDE surface, the surface was first modified to form an amine by APTES. For that, 2% APTES was diluted in 30% ethanol, dropped on the sensing surface and incubated for a minimum period of 1 h at RT. After 1 h, the surface was washed five times using a pipette with 30% ethanol followed by water, and then 1 μl of prepared GNR–aptamer-conjugated probe was dropped onto the surface. Then, the remaining sensing surface was blocked by 1 M ethanolamine to prevent nonspecific adsorption of cortisol on the sensing area. Between each modification/interaction, the surface was washed thoroughly five times

with five volumes of PBS (10 mM; pH 7.4). All steps were performed under wet conditions throughout the experiments.

Cortisol detection on the GNR–aptamer-modified IDE surface

Cortisol was detected on the GNR–aptamer-modified surface by initially adding 1000 ng/mL cortisol to the probe-immobilized surface. Changes in the current were monitored using an ammeter to detect the cortisol. To determine the limit of detection, different concentrations (0.01–100 ng/mL) of cortisol were dropped independently on the GNP-aptamer-modified IDE surface from the lowest to the highest concentrations. Changes in the current were monitored for each experiment after thoroughly washing the surface with 10 mM PBS (pH 7.4). Experiments were performed in triplicate, and average values were calculated. A linear sweep voltage of 0–2 V at a 0.01 V step was applied for the measurements. The limit of detection (LOD) was defined as the lowest concentration of an analyte (from the calibration line at low concentrations) detectable against the background signal ($S/N = 3:1$); in other words, $LOD = \text{standard deviation of the baseline} + 3\sigma$.

Specific detection of cortisol on a GNR–aptamer-modified IDE surface

To confirm the specific detection of cortisol, control experiments were carried out with two different hormones: nor-epinephrine (control 1) and progesterone (control 2). High concentrations (1 μg/mL) of these hormones were added independently to the GNR–aptamer-modified surfaces and compared with the 1 μg/mL cortisol interaction. Changes in the current were recorded for each experiment after the washing steps using 10 mM PBS (pH 7.4). Experiments were performed in triplicate, and average values were calculated. A linear sweep voltage of 0–2 V with a 0.01 V step was applied for the measurements. Between each measurement, the surface was washed thoroughly five times with five volumes of PBS (10 mM; pH 7.4). All steps were performed under wet conditions throughout the experiments.

Cortisol detection in human serum

Cortisol was detected in commercial human serum on a GNR–aptamer-modified IDE surface. For that, diluted human serum samples (as-received serum, 1:10, 1:100, 1:1000) were applied to GNR–aptamer-modified IDE surfaces for 30 min at RT. After washing the surface, changes in the current were recorded.

Results

Conjugation and immobilization of the GNR–aptamer

Figure 1a shows a schematic representation of cortisol detection by the aptamer-GNR probe on the IDE surface. As shown in the figure, first, the surface was modified by APTES to immobilize the GNR–aptamer, and then cortisol was detected on the aptamer-GNR-modified IDE surface. Here, we immobilized the aptamer on a gold surface through a thiol-linker and captured the amine tethered surface, as amines have affinity to gold. A 1 μM solution of aptamer was conjugated with GNR and immobilized on an APTES-assisted sensing surface. This study limited the aptamer concentration to 1 μM to leave free space on the GNR for attachment onto the amine surface. When the aptamer is conjugated to GNR in the solution state, the aptamer binds to the GNR surface with a properly aligned exposed region and can easily recognize low levels of cortisol. After attaching the molecules on the IDE surface, the electrode (finger) and gap regions will be connected, creating a dipole moment upon application of a current (Fig. 1b).

Surface morphology of an IDE sensing surface

The surface of the IDE was analyzed by high-power microscopy and 3D profilers. To make a proper comparison, we observed the surface profile in both the absence and the presence of GNR–aptamer attachments. Figure 2a displays the surface morphology of the bare IDE, showing the gap and electrode regions. Figure 2b clearly illustrates the presence of immobilized particles on the surface of the IDE and the exclusive binding on the gap regions. Because of APTES

modification on the gaps, these regions were attracted by the GNR–aptamer conjugates. Similarly, we also observed the surface by high-power microscopy. Figure 2c shows the obtained image along with a scanned profile generated by means of ImageJ software (<https://imagej.nih.gov/ij/download.html>).

Analysis of the secondary structure of the aptamer

Before analyzing the interaction between GNR–aptamer and cortisol, we predicted the secondary structure of the anti-cortisol aptamer by means of mfold software. In the case of the anti-cortisol aptamer, we observed three possible structures: structures 1 and 2 show 4 loop regions and 3 stems (Fig. 3a, b), whereas structure 3 has 5 loop regions and 4 stems, highlighting the possibility for varied structures (Fig. 3c). The comparative analysis also clearly indicates the possibility of 3 different structures involving cortisol and the aptamer; however, the energy levels (dG) of all the structures are similar (Fig. 3d).

Electrical characterization of the molecular assembly on an IDE surface

Figure 4a presents electric measurements of the GNR–aptamer conjugate and other molecular immobilizations on the IDE surface. As shown in the figure, only the bare surface shows a current level of 7.65 E^{-07} ; after attaching APTES, the current was increased to 9.56 E^{-05} . Then, upon immobilizing the GNR–aptamer conjugate, the current level was decreased to 3.14 E^{-07} , which might be due to the higher changes in the surface charge upon formation of the GNR–aptamer complex. These changes confirmed the immobilization of the aptamer-GNR conjugate on the amine-modified surface. After that, the remaining free

Fig. 1 **a** Schematic representation of cortisol detection by an aptamer-gold nanorod probe on an IDE surface. The surface was modified by APTES to immobilize the GNR–aptamer, and cortisol was detected on the aptamer-GNR-modified IDE surface. **b** Gap regions on the surface of the IDE sensor are displayed. When the electrode (finger) and gap regions are connected, a dipole moment is generated upon application of a current

Fig. 2 **a** Surface morphology of bare IDEs, showing the gap and electrode regions. **b** Illustration of the presence of immobilized particles on the surface of the IDE and exclusive binding to the gaps. **c** Image

of the IDE sensing surface observed by high-power microscopy along with the scanned profile obtained by means of ImageJ software

surface was blocked by ethanolamine to avoid nonspecific binding of cortisol. When we added ethanolamine, the current level was increased to 2.43×10^{-4} . This aptamer-GNR conjugated sensing surface was considered the complete sensing area to detect cortisol.

Interaction between GNR–aptamer and cortisol

To evaluate the interaction between GNR–aptamer and cortisol, initially, a high concentration of cortisol ($1 \mu\text{g/mL}$) was added onto the aptamer-GNR immobilized surface, and interaction between the aptamer and cortisol was clearly observed by the decrease in the current level from 2.43×10^{-4} to 1.23×10^{-5} (Fig. 4b). This is a decrease of approximately 20-fold in the level of current due to the binding of the aptamer and cortisol. The obvious changes with different molecular immobilizations are displayed in the figure inset. Since $1 \mu\text{g/mL}$ cortisol was clearly detected, we next titrated different amounts of cortisol to determine the limit of detection. The cortisol was diluted to the low nanogram range and applied onto the GNR–aptamer-modified surface. As shown in Fig. 5a, with an increase in the cortisol concentration, the level of current gradually decreased due to the binding of cortisol to the aptamer. The green line represents the binding of ethanolamine (2.43×10^{-4}); after adding the lowest

concentration of cortisol (0.01 ng/mL), the current level was decreased to 1.37×10^{-4} (black line). Then, as the concentration of cortisol increased, the current level gradually decreased. This result is clear evidence for cortisol binding to the aptamer. Figure 5b presents the difference in the current relative to that for ethanolamine at each concentration of cortisol tested. It was clearly observed that with increasing cortisol concentration, the difference increased. The limit of detection of cortisol was found to be 0.01 ng/mL .

High-analytical performance for sensing cortisol by the GNR–aptamer

To estimate the sensitivity level, we generated a linear plot of the interaction of different levels of cortisol by plotted the response obtained from 0.01 to 1000 ng/mL cortisol. Based on 3σ , we calculated the sensitivity range to be between 0.01 and 0.01 ng/mL (Fig. 6a). We also validated and plotted the reproducibility of the current level with bare, APTES-modified, GNP-aptamer-modified, ethanolamine-modified and cortisol-bound surfaces (Fig. 6b). These results confirm the high reproducibility levels of the sensing surface with different modifications.

To determine the specific interaction of cortisol on the GNR–aptamer-modified IDE surface, control experiments

Fig. 3 Possible secondary structures of the cortisol aptamer. Structures 1 (a) and 2 (b) show 4 loop regions and 3 stems; c Structure 3 has 5 loop regions and 4 stems, highlighting the possibility for varied

structures. d Comparison and energy levels (dG) of all the structures obtained with the anti-cortisol aptamer

were carried out with two different hormones: norepinephrine and progesterone. It was found that only cortisol could interact specifically with the aptamer. The other

hormones did not show any significant changes in the current after interaction (Fig. 7a). Among these control hormones, progesterone displayed slight binding compared

Fig. 4 **a** Probe prepared by GNP-aptamer immobilization on the IDE sensing surface. APTES was used to modify the IDE surfaces. GNR-aptamer was immobilized, and then the remaining surfaces were blocked by ethanolamine. A schematic representation is shown in the

figure inset. **b** Cortisol detection on the GNP-aptamer-modified IDE sensing surfaces. The figure inset displays the differences in current levels upon immobilizing the molecules

Fig. 5 **a** Dose-dependent binding of cortisol on the GNR-aptamer-modified IDE sensing surface. Cortisol from 0.01 to 1000 ng/mL was detected on the IDE surface, and the limit of detection was deter-

mined to be 0.01 mg/mL. A schematic representation is shown in the figure inset. **b** Decreases in the current level when the concentration of cortisol was increased

to norepinephrine. However, this binding was negligible compared to the specific binding of cortisol. Only cortisol binding resulted in a 20-fold change in the current, which indicates the specific detection of cortisol by its aptamer (Fig. 7a, inset).

After confirming the specific detection of cortisol, cortisol was also detected in human serum. Diluted human serum was applied onto GNR-aptamer modified surfaces, and changes in the current were monitored, as shown in Fig. 7b. After adding a 1:1000 dilution of human serum, clear changes were monitored. With a decrease in the dilution

to 1:100 and 1:10, the current levels were further changed, indicating the detection of cortisol in human serum.

Discussion

Cortisol is a stress hormone, and an optimum level of cortisol needs to be maintained for good human health. Abnormal levels of cortisol cause various health complications, including hypoglycemia and hypertension; therefore, it is mandatory to quantify the levels of cortisol for efficient

Fig. 6 High-performance analysis. **a** Linear graph of the concentration-dependent binding of cortisol. The sensitivity region is indicated based on a 3σ calculation. **b** Reproducibility of different surface modifications on different fabricated devices

Fig. 7 Selectivity analysis. **a** Control experiments with two different hormones: norepinephrine and progesterone. The anti-cortisol aptamer recognized only cortisol, while the other hormones did not show any obvious changes in the current. The figure inset displays the binding levels of cortisol and other hormones on the GNR-aptamer-

modified surfaces. A schematic representation is shown in the figure inset. **b** Detection of cortisol in the serum samples. Diluted serum interacted with GNR-aptamer-modified surfaces, and changes in the current were recorded. Changes in the current clearly indicated the presence of cortisol in all the concentrations of serum samples

treatment. In the past, various probes and sensing methods have been used to detect cortisol. In general, an antibody is used to detect cortisol in biofluids (Jang et al. 2018). In this work, we designed a new approach for the anti-cortisol aptamer-based detection of cortisol, and aptamer sequences were adapted from a previous study (Sanghavi et al. 2016). The aptamer used is quite stable because it consists of DNA sequences. Furthermore, it is a short, synthetic molecule that is stable under stringent conditions. In general, aptamers are highly selective and sensitive to their target, showing a high detection level. In addition, aptamers are more stable than antibodies, so they are easier to conjugate with nanomaterials (Lakshmipriya et al. 2013a, 2016b). Nanomaterial-conjugated aptamers have been shown to improve detection

with high performance. Different nanomaterials, such as gold, silver, graphene, titanium and iron, have been used in biosensors to enhance the limit of detection (Gopinath and Kumar 2014). Among these metals, gold is a popular choice for surfaces, and gold nanoparticles have been utilized in various sensing technologies, including surface plasmon resonance (Gopinath and Kumar 2014), waveguide mode sensing (Lakshmipriya et al. 2013b), electrochemical sensing (Letchumanan et al. 2019), enzyme-linked immunosorbent assay (ELISA) (Lakshmipriya et al. 2016a) and colorimetry (Gopinath et al. 2014). In this study, we prepared a probe (aptamer)-conjugated gold nanorod (GNR) on an interdigitated electrode (IDE) surface to detect a stress hormone (cortisol) for the diagnosis of hypoglycemia and hypertension.

In general, the secondary structure of the aptamer consists of stem and loop regions that interact with the target. Usually, the stem is double-stranded, increasing the stability of the structure, whereas the loop is a single-stranded region that tends to interact with the target. The GNR–aptamer conjugates were stabilized with anionic citric ions that can bind to the cationic APTES by electrostatic interactions (Liu et al. 2012; Williams et al. 2013). If we immobilize only aptamers on the chemically modified sensing surfaces, there is less chance of interaction with cortisol due to the limited number of aptamers on the sensing surface. However, aptamer-conjugated GNRs have many aptamers, allowing the probe to interact efficiently with cortisol. In addition, in any biomolecular immobilization on a sensing surfaces, orientation plays a crucial role in detection. It has been proven that the proper orientation of biomolecules by polyethylene glycol-based polymers on a gold surface improved the detection of blood clotting FIX protein (Uchida et al. 2005; Anniebell and Gopinath 2018). This study quantified the levels of cortisol with the assistance of aptamer-conjugated gold nanorods on an electrochemical IDE sensor and achieved a detection limit of 0.01 ng/mL. Moreover, a specificity experiment was carried out with the control hormones norepinephrine and progesterone, and it was found that the aptamer specifically recognizes only cortisol. It is interesting to note that the current strategy is also able to detect cortisol in serum samples. The normal range of cortisol in the serum of healthy humans is 100–200 ng/mL. In our experiment, cortisol was detected in as-received serum and diluted serum samples (1:10, 1:100, and 1:1000), which are equivalent to 20, 2 and 0.2 ng/mL cortisol. This detection is clearly beneficial to the diagnosis of both hyperglycemia and hypertension during pregnancy.

Conclusion

Cortisol is a stress hormone released from the adrenal glands and has been shown to be responsible for both hyperglycemia and hypertension during pregnancy. When the level of cortisol is decreased in circulating blood, the liver faces difficulty in converting stored blood sugar (glycan) into active blood sugar (glucose). At the same time, a high level of cortisol is responsible for gestational hypertension. The current study on aptamer-mediated quantification of cortisol for the medical diagnoses of multiple pregnancy-related diseases showed good sensitivity and selectivity. The detection limit was 0.01 ng/mL, and the sensitivity ranged from 0.01 to 0.1 ng/mL. Furthermore, this system was able to discriminate against closely related hormones (norepinephrine and progesterone) and to detect cortisol in serum. This method is useful to detect and correlate multiple pregnancy-related diseases by identifying the level of cortisol.

Author contributions The authors all contributed to the preparation of the manuscript and discussion. Both authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest On behalf of all the authors, the corresponding author states that there is no conflict of interest.

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