An electrochemical biosensor for the determination of hormone Human Chorionic Gonadotropin (hCG) in human serum

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Abstract

This work describes the modification of a gold electrode to create an electrochemical biosensor capable of detecting human chorionic gonadotropin (hCG). The biosensor was obtained by modifying the gold electrode with cysteamine and oligopeptide (PPLRINRHILTR). The modification steps of the gold electrode were confirmed by cyclic voltammetry (CV) and impedance electrochemical spectroscopy (EIS) measurements. The conducted EIS experiments in 0.01 M PBS, pH 7.4 confirm that the biosensor exhibits sensitivity towards hCG in a range of concentrations from 1×10^{-12} to 1×10^{-7} M (0.5 mIU/mL – 50 000 mIU/mL) to solutions with a detection limit of 1.91×10^{-14} M (0.0095 mIU/mL). The effectiveness of the investigated biosensor was also investigated in human serum. The EIS comparative investigations were performed in human serum containing a concentration of 1×10^{-12} M (0.5 mIU/mL) hCG and in human serum where the hCG was added. The obtained results indicate that the investigated biosensor is selective for the presence of hCG hormone in the human serum.

Keywords: Human Chorionic Gonadotropin (hCG), gold electrode functionalization, human serum, electrochemical detection,

1. Introduction

Human chorionic gonadotropin (hCG) is a 37 kDa glycoprotein hormone consisting of two combined, dissimilar subunits designated alpha (common to other glycoprotein pituitary hormones - LH, FSH, TSH) and beta, which are specific for hCG [1] It has 8 sites of glycosylation – 4 N and 4 O; because of their different glycosylation their weight varies from 27.6 to 42.8 kDa [2]. There are at least 18 different isomers or degradation products which are biologically active. The hCG is the most glycosylated hormone in humans – the percentage of the sugars'weight varies from 28 to 42 % [3]. The consequence of such a high sugar component is a very long half-life in human blood which could last 36 hours [4].

hCG is the essential pregnancy hormone, necessary for syncytiotrophoblast formation, embryo implantation, placental development and pregnancy maintenance. It stimulates progesterone production, causes quiescence of the myometrium and angiogenesis. Production of the hCG starts before implantation probably during the blastocyst formation [5].

Early hCG secretion is a marker of good embryonic development and indicates a good prognosis for the development of the pregnancy. It is therefore important to be able to accurately measure hCG as early in pregnancy as possible, when its levels are very low. The average serum level of hCG

in a healthy pregnancy in the third week is about 0.26 ng/ml and in the peak period of its secretion it exceeds 10000 ng/ml [4]. hCG is also a factor involved in the pathomechanism of many pregnancy complications.

The deficiency of hyperglycosylated hCG (the main form of hCG in early pregnancy) is considered to be the main factor of hypertensive pregnancy [6], pre-eclampsia, or the development of pregnancy induced hypertension (PIH), but also predicts poor hemochorial placentation growth or nutritional deficiency. The hCG level is also used in prenatal diagnostics for Down syndrome pregnancies risk estimation [7].

Attempts to determine hCG levels have been made since its role was first understood. Biological tests have been carried out since 1927 – using urine injected into mice (1927), rabbits (1931), toads (1934) or rats (1941) [8].

Electrochemical determination of the hCG hormone is most often performed using antibodies on modified electrodes [2,9–14]. There are some literature reports where instead of expensive antibodies, oligopeptide was used that interacts with hCG. The above works are mainly based on the short oligopeptide sequence (PPLRINRHILTR), which was selected from a phage library after five rounds of screening reported by Ding and Yang [11]. The above oligopeptide was previously used for the detection of hCG. Based on the mechanism of the catalytic properties of gold nanoparticles (AuNPs) with described peptide, however, a label-free colorimetric assay was designed for the detection of hCG [15]. The graphene oxide (GO) with a oligopeptide modified by fluorescein isothiocyanate was employed for the detection of the hCG [16]. GO sheets modified by oligopeptides placed on the modified gold were also applied for an ultra-high hGC sensitive biosensor based on surface plasmon resonance (SPR) changes [17]. The oligopeptide used for the hGC detection also utilized silver nanoparticles (AgNPs) as redox reporters employing linear-sweep voltammetry (LSV) [18].

Furthermore, one of the most commonly used methods for the detection of hCG is the application of hormone-specific anti-hCG antibodies. Modifications with anti-hCG usually can be performed on a GC electrode modified with Pt nanoparticles and graphene ionic liquid chitosan nanocomposite [19], modified with silver nanoparticles and a nanocomposite composed of graphene, chitosan and ionic liquid, using riboflavin as a redox probe [20] and a GC electrode modified by gold nanoparticles and cysteamine [21].

In this work, we demonstrate a new approach of the (PPLRINRHILTR) oligopeptide to obtain a novel, modified gold electrode sensitive to the presence of hCG both in a phosphate buffer solution (PBS) used as a reference solution and in a human serum solution. The sensitivity of the prepared gold electrode was investigated in a 0.01 M PBS solution in a concentration range from 1×10^{-12} to 1×10^{-7} M (0.5 mIU/mL – 50 000 mIU/mL) using the (EIS) method. The efficiency of the obtained electrodes was also tested in human serum. The comparative hCG detection was performed in a human serum of the concentration 1×10^{-12} M (0.5 mIU/mL) and in human serum where the hCG was added. The obtained results demonstrate that the obtained biosensor is sensitive for hCG in the PBS solution and human serum samples. The hCG detection is extremely important in the assessment of the normal course of a pregnancy and the diagnosis of many cancers including trophoblastic and nontrophoblastic cancers such as gynaecological cancers, testicular cancers or biliary and pancreatic cancer and many others [1,22,23]. The invention of a biosensor that works in a variety of media including human serum is very crucial.

2. Experimental

2.1. Materials and reagents

All of the chemicals were of an analytical grade. N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), piperidine, N,N-Diisopropylethylamine) (DIPEA), N,N′-Diisopropylcarbodiimide (DIC) Ethyl(hydroxyimino)cyanoacetate (Oxyma), Trifluoroacetic acid (TFA), Triisopropyl silane (TIPSI), Acetonitrile (ACN) cysteamine, bovine serum albumin (BSA), $K_3[Fe(CN)_6]$, and $K_4[Fe(CN)_6]$ were purchased from Sigma-Aldrich. 0.01 M PBS, pH 7.4 was obtained from tablets purchased from Sigma-Aldrich. 0.02 M PBS, pH 5.15 was obtained from (2.72 g KH_2PO_4, adjusted to suitable pH by KOH in 1000 mL). Alumina powders of 0.03 μm and 0.05 μm and microcloth pads were purchased from Buehler (USA). Ethanol, N, and N-dimethyloformdamide (DMF), were purchased from POCh (Poland).

2.3. Peptide synthesis (PPLRINRHILTR)

The (PPLRINRHILTR) (Pro-ProLeu-Arg-Ile-Asn-Arg-His-Ile-Leu-Thr-Arg) oligopeptide was synthesised applying the standard solid-phase peptide synthesis (SPPS) protocol using Fmoc/tBu chemistry on an automated microwave synthesizer,

Liberty BlueTM (CEM Corporation, USA). The oligopeptide used for electrode surface modification was obtained with the Fmoc protecting group at the N-terminus of amino acid. Synthesis was performed on a 2-chlorotrityl chloride resin with the capacity of 1.0-1.6 mmol/g (Lipopharm, Poland) using standard amino acids derivatives. The coupling of the C-terminal arginine residue was performed manually prior to synthesis on the Liberty BlueTM with a two fold excess of N-Fmoc protected arginine with the addition of a four fold excess of DIPEA (N,N-Diisopropylethylamine). Fmoc protecting groups were removed by the use of a 20% piperidine solution in DMF. The coupling reactions run on the Liberty BlueTM were performed with a 0.5 M DIC solution in DMF with a 1 M solution of Oxyma pure in DMF. The cleaved processes were run with the simultaneous removal of amino acids side chains protecting groups in the mixture of 88% TFA/5% phenol/5% H_2O/2% TIPSI. The crude peptide was purified using the reverse-phase high performance liquid chromatography (RP-HPLC) technique on a semi-preparative column – Luna C8 (2) (250 mm x 20 mm, 5 μm) from Phenomenex (Torrance, USA), in an ambient temperature using an appropriate H_2O/ACN gradient with 0.1 % of TFA. The purity of the final product was confirmed by RP-HPLC using the analytical column Kromasil C8 (250 mm x 4.6 mm, 5 μm) and mass spectrometry.

2.4. Preparation of the hCG electrochemical biosensor

The hCG electrochemical biosensor presented in this paper is based on a chemical modification of the gold electrode. In the first step, the gold electrodes were modified by immobilization by covalent linkage via cysteamine, performed according to the literature procedure [24]. The 18 mM cysteamine solution in ethanol, during 12 h was used to obtain a self-assembled monolayer (SAM) on the electrode's surface.

In the second step, the oligopeptide (PPLRINRHILTR) was attached to the amino groups present on the gold electrode using the EDC/NHS mixture solution as coupling agents. The cysteamine modified electrodes were placed in a 0.02 M PBS solution of pH 5.15 containing 1.5 mM of oligopeptide in a form of (Fmoc-PPLRINRHILTR), containing the Fmoc group as a protecting group of the N-chain and 100 mM EDC and 50 mM NHS. The reaction was performed in room temperature in a 200 μl vessel over 16 h. Then the electrodes were placed in a 200 μl vessel containing a 20 % solution of piperidine in DMF during 30 min to remove the Fmoc group. The Fmoc group removal reaction was repeated twice.

Next the electrodes were rinsed with 0.01 M PBS, pH 7.4 and deionized water, and incubated in 10 μL of a 0.1% (BSA) solution for 45 min to block the unreacted and non-specific sites. The electrodes obtained in such a manner were subsequently applied for the detection of the (hCG). The detection of the (hCG) hormone was performed (0.01 M (PBS) buffer, pH 7.4, and human serum) by applying 10 μL on the electrode's surface for 45 minutes. The sensitivity of the gold electrodes to hCG in different concentrations were evaluated by EIS measurements.

2.5. Serum extraction

Human serum was obtained from anonymized patient samples collected for routine clinical analysis. These were human serum samples from subjects gestation period with known hCG levels established by an electrochemiluminescence assay on roche cobas performed on Elecsys total HCG cobas, (Roche Switzerland).

3. Results and discussion

3.1. Functionalization of the gold electrode for the hCG detection

The structures of both bovine serum albumin (BSA) and hCG presented in papers [25,26], were used to schematically represent the various steps of the electrode modification. A chemical modification scheme for the gold electrode is shown in Fig.1.

The first modification of the gold electrode was carried out in an 18 mM solution of cysteamine in anhydrous ethanol over 12 h, applying the procedure described elsewhere [24].The monolayer based on the cysteamine in ethanolic solution is a commonly used technique in the preparation of biosensors and allows for the gold's modification [27,28].

The application of (SAM) allows for the coating of a surface that can be further chemically modified to obtain biosensors [29]. The usage of cysteamine allows for the amino groups presence on the electrode's surface. The oligopeptide was ahnored on the gold electrode through the activation of carboxyl groups in the presence of a mixture of EDC/NHS. The reaction was performed in a phosphate buffer solution pH 5.15, due to the fact that the EDC/NHS reaction is the most efficient in the pH range of 4.5 to 7.2 [30].

Then the electrodes were placed in a solution of 0.1 % of BSA which is used to prevent non-specific adsorption on the surface of the electrode and to block non-specific binding sites [31]. The sensitivity of the obtained electrochemical biosensors were examined to detect the hCG hormone in the PBS solution in various concentrations from 1×10^{-12} to 1×10^{-7} M (0.5 mIU/mL – 50 000 mIU/mL) used as the standard solution as well as in human serum containing a known concentrattion of hCG (1×10^{-12} M) (0.5 mIU/mL) and in human serum with the addition of hCG. The electrochemical changes of electrodes during the functionalizastion process were measured by the CV and EIS methods, while in the presence of hCG involved different media were monitored using the EIS techniques.

3.2. Electrochemical investigations of the electrode during the biosensor formation

3.2.1. Cyclic voltammetry

CV and EIS measurements were performed after each gold electrode's functionalization step were carried out in a 0.01 M PBS solution containing 1 mM of $([Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ used as redox couples. This redox mediator belongs to inner-sphere electrochemical redox probes sensitive to electrode surface changes [32,33]. Fig. 2a shows the CV's obtained for the bare gold electrode, modified with cysteamine, peptide, and the response of the electrode in the presence of hCG in the concentration of 1×10^{-7} M (50 000 mIU/mL).

The quasi-reversible redox process is observed for the bare Au electrode with peak to peak separation $\Delta E = 90$ mV. For the modified electrode with the cysteamine, the peak to peak separation decreased to 74 mV. This indicates the self-assembly monolayers (SAM's) formation on the electrode's surface. The presence of protonated amine groups on the surface enhances the transfer of electrons [34], which interact with the applied redox probes [35].

The presence of oligopeptide on the electrode's surface slightly influence the electrode's response. The modification by oligopeptide does not affect the observed voltammetric currents, but the peak to peak separation also increases from 74 mV to 81 mV.

This is probably attributed to the chemical structure of the attached oligopeptide, which processes positively charged amino acids in the structure.

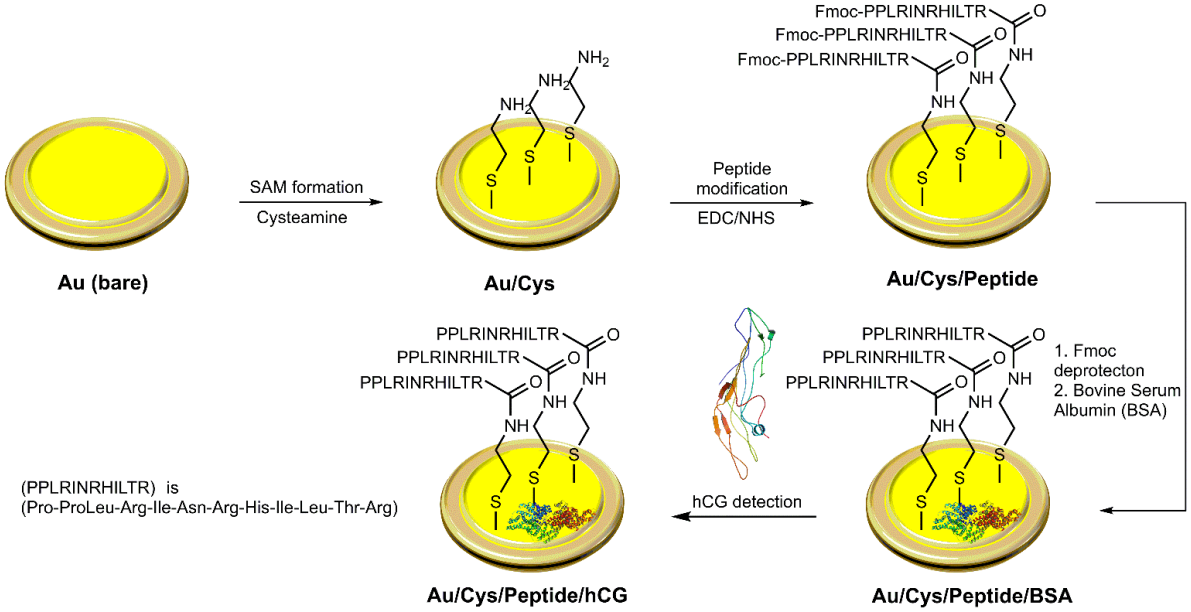

Fig. 1. The functionalization scheme of a gold electrode as an electrochemical biosensor for hCG detection.

The electrochemical response of the investigated biosensor was tested in a 0.01 M PBS solution containing 1×10^{-7} M (50 000 mIU/mL) of hCG by CV which resulted in the peak-to-peak separation increasing from 81 mV to 179 mV; moreover, a significant decrease of the height currents were observed on cyclic voltammeters (see Fig. 2a).

3.3.2 Electrochemical Impedance Spectroscopy

The EIS measurements were performed both for the characterization of the electrodes at each stage of their functionalization and for the hCG detection. EIS is a high-sensitivity measurement method

frequently used to characterise the various steps of the electrode modification of biosensors including the (SAM's) formation [39]. The EIS measurement method gives information that cannot be achieved by CV. Therefore, EIS studies are often used to investigate the phenomena occurring at the electrode's surface during the binding of the analytes of many biosensors [37].

All of the obtained impedance spectra in this paper were fitted using the modified Randles equivalent circuit consisting of: solution resistance (R_s), constant phase element (CPE), electron transfer resistance (R_{ct}) and Warburg impedance element (W) (see inset of Fig. 2b).

The CPE in the applied equivalent circuit model, including irregularity of the electrode's surface and inhomogeneity of the current distribution, was used instead of double layer capacitance [38]. The description of CPE including impedance analysis of electrode materials and biosensors is described in many papers [39–42].

The obtained EIS measurement data here are presented as points, while lines show the fitted data. The calculated value from the fittings for all the obtained experimental data are presented in Tables S1, S2 and S4, where it can be seen that the applied equivalent electrical circuits were well fitted, as indicated by the Chi^2 parameter.

The impedance spectra for all the steps of the modification of the gold electrode are shown in Fig. 2b. The calculated R_{ct} value for the bare Au electrode is 986.9 Ω. This value decreases to 337.8 Ω after the (SAM's) formation. The presence of the positively charged amino group on the electrode's surface which interacts with negatively charged redox probes caused the increases of the charge transfer to the electrode [43].

The immobilization reaction of oligopeptide containing the Fmoc group resulted in a decrease of R_{ct} value to 305.2 Ω. The removal of the Fmoc group from and incubation in 0.1% BSA solution caused the decrease of the R_{ct} value to 289.9 Ω indicating a slight effect of BSA on the electrode's surface.

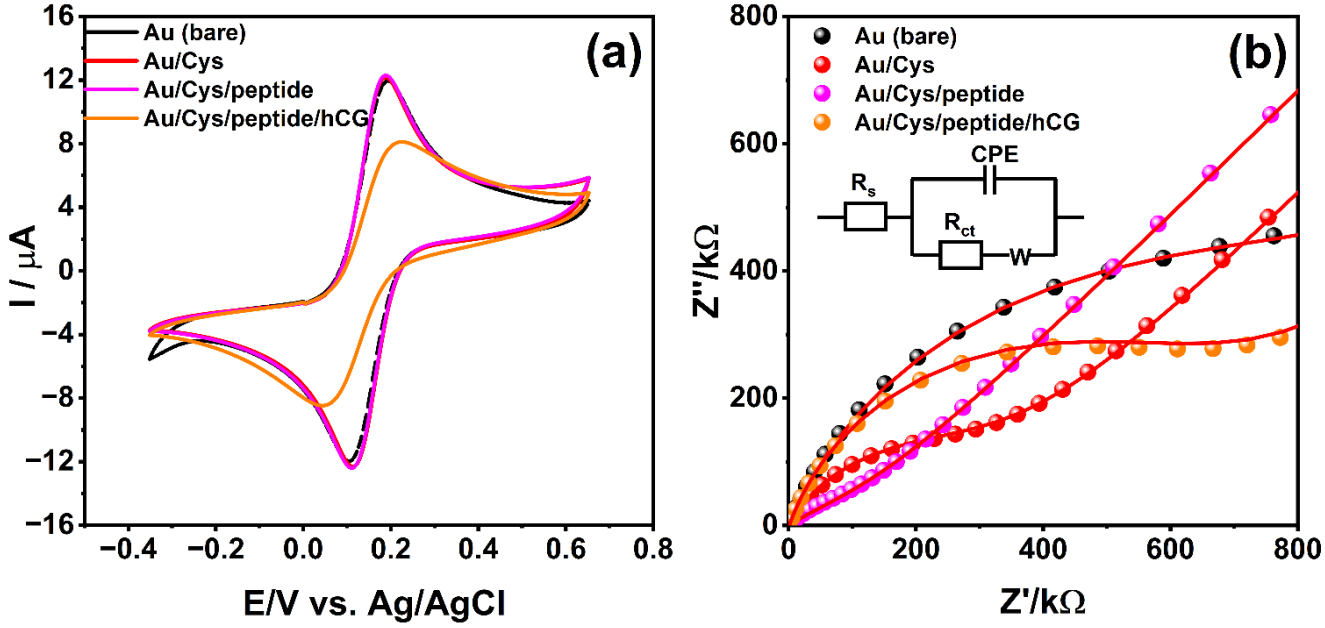

Fig. 2. a) CV, b) EIS curves obtained for: Au bare, Au modified with cysteamine, Au modified with cysteamine and oligopeptide and response of obtained electrodes in the presence of hCG in concentration of 1×10^{-7} M (50 000 mIU/mL) in 0.01 M PBS, pH 7.4 containing 1 mM $Fe[(CN)_6]^{3-/4-}$.

The obtained electrode was utilized to 1×10^{-7} M (50 000 mIU/mL) hCG detection in the PBS solution (see Fig. 2b). The incubation in this solution caused the appearance of a semicircle in the impedance spectra and increased the R_{ct} value to 1014.0 Ω indicating an inhibition of electron transfer. The presence of hCG results in an increase of R_{ct} by 3.5 times.

The analysis of the EIS data also reveals very interesting changes in the α parameter. The parameter α for the cysteamine modified electrode is about 0.8, which indicates a certain roughness (for the smooth electrode α =1 [44]) or inhomogeneity of the charge distribution [45,46]. The attachment of an oligopeptide to the electrode causes a change in roughness and a decrease of parameter α to 0.49. Nevertheless, the attachment of the hCG hormone causes an increase in the α parameter to about 0.87 for the hCG concentration of 1×10^{-7} M (50 000 mIU/mL). Further investigated hCG

concentrations in the range from 1×10^{-12} to 1×10^{-7} M (0.5 mIU/mL – 50 000 mIU/mL) result in a linear decrease of the α parameter from 0.87 to 0.82 respectively. The detailed results of EIS analysis are presented in Table S1

3.3. Electrochemical hCG hormone detection in PBS solution

The sensitivity of the obtained biosensor was tested in a 0.01 M PBS, pH 7.4 used as a standard solution in hCG concentrations of from 1×10^{-12} to 1×10^{-7} M (0.5 mIU/mL – 50 000 mIU/mL). The electrodes were incubated at the biosensor's surface for 45 min in solution, then the EIS measurements were performed (see Fig. 3a.)

The obtained R_{ct} values indicate that the R_{ct} increases according to the hCG concentration increase after incubation (see Table S2). The ΔR_{ct} was calculated for equations of the R_{ct} value of the electrode after incubation in suitable human hCG concentration and the R_{ct} of the electrode after incubation in BSA. Based on the dependence of ΔR_{ct} on the logarithm of the hCG concentration (Figure 3b), two linear regression equations were observed from 1×10^{-12} to 1×10^{-9} M (0.5 mIU/mL – 500 mIU/mL) and 1×10^{-9} to 1×10^{-7} M (500 mIU/mL – 50 000 mIU/mL) with the following equations: $\Delta Rct = 64.50 \log C(hCG)\ (M) + 778.99$ with $R^2 = 0.987$ and $\Delta Rct = 216.41 \log C(hCG)\ (M) + 2199.34$ with $R^2 = 0.991$ respectively.

The calculated detection limit (LOD) of the hCG biosensor is 1.91×10^{-14} M (0.0095 mIU/mL), estimated on the basis of the detection analysis limit, presented elsewhere [47,48].

The detection limit of the hCG of the sensors based on the described oligopeptide were: 0.065 nM for SPR [17], 15 mIU/ml and 25 mIU/ml for colorimetric assay[15], [18], 20 mIU/ml for fluorescent measurement [16] and 1 IU/ml for liquid crystals [11]. The results obtained for the described biosensor allows for the hCG detection at comparable or lower concentrations. Due to the fact that 1 IU/mL is 2 nM [11], the limit of the detection of hCG determination is 1. 91 $\times 10^{-14}$ M (0.0095 mIU/mL). Additionally the above method of hCG detection with others based on electrochemical techniques including the synthetic oligopeptide usage [49–52] are compared in Table S3 .

3.4. Detections of hCG hormone in Human Serum

The investigated biosensor has been also investigated in human serum which was selected due to the fact that clinical assays for hCG determination are usually performed in this medium. The EIS investigations were performed in human serum containing hCG ranging from 1×10^{-13} to 1×10^{-11} M (0.05 mIU/mL – 5 mIU/mL) . Only the results for the 1×10^{-12} M (0.5 mIU/mL) are presented in this work due to the fact that the lower concentrations of hCG were not possible to detect.

The obtained EIS spectra shows the biosensor response recorded in solutions of: human serum without hCG hormone (blank solution), reference human serum containing 1×10^{-12} M (0.5 mIU/mL) hCG and human serum (blank solution) with 1×10^{-12} M (0.5 mIU/mL) hCG addition (see Fig. 4). The detailed results of the EIS analysis spectra are presented in Table S4 in (SI).

The obtained R_{ct} value for the biosensor after incubation in human serum used as a blank solution was equal to 140,6 Ω, while the R_{ct} value for the human serum containing 1×10^{-12} M (0.5 mIU/mL) hCG increased to 328.1 Ω.

In order to confirm the hGC detection effectiveness of the tested biosensor the electrode was incubated in human serum (previously used as a blank solution) where the 1×10^{-12} M hCG (0.5 mIU/mL) was added. The R_{ct} value calculated for the biosensor in this solution is equal to 349.1 Ω, which is close to the R_{ct} value calculated for the biosensor response in the solution containing the same concentration of hCG which is equal to 328.1 Ω.

This experiment shows that the obtained biosensor is able to detect the hCG hormone in human serum by the EIS method.

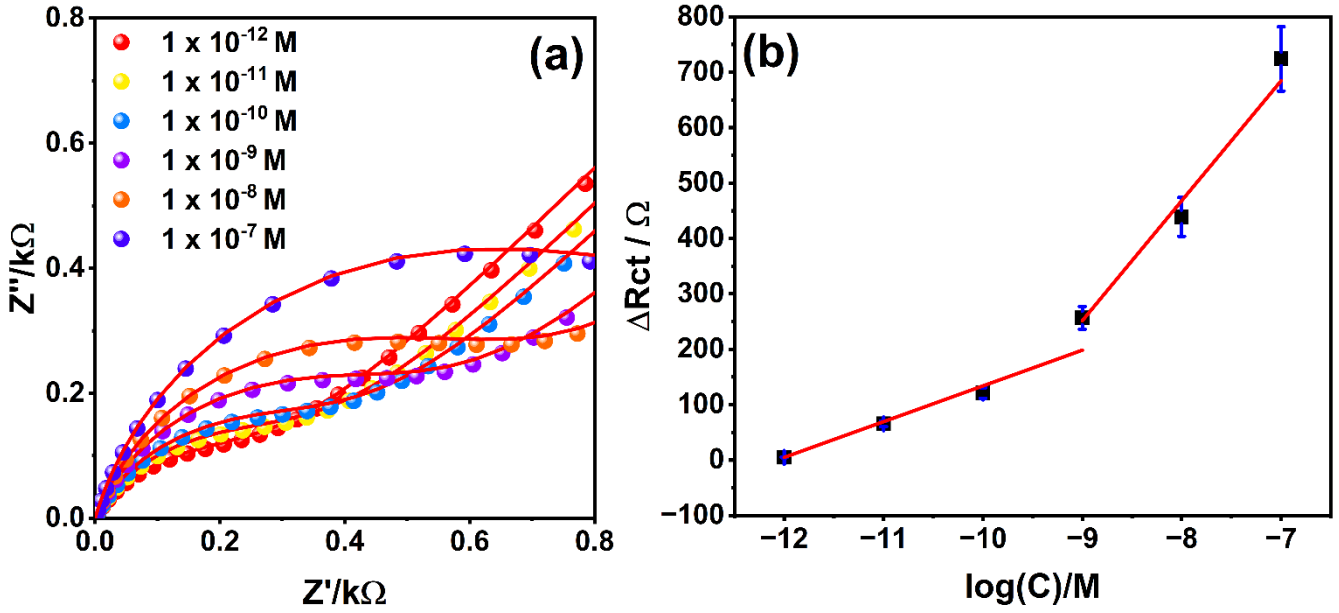

Fig. 3. a) Electrochemical impedance spectroscopy curves obtained after incubation at a concentration of hCG hormone ranging from 1×10^{-12} to 1×10^{-7} M (0.5 mIU/mL – 50 000 mIU/mL) in 0.01 M PBS. b) Dependence between the calculated ΔR_{ct} and hCG concentrations.

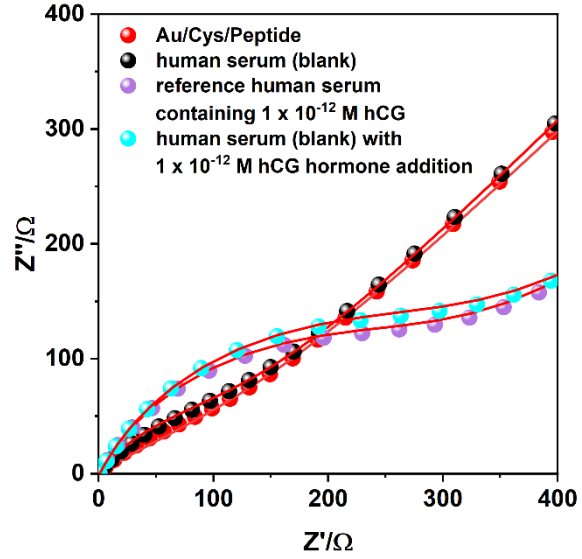

Fig. 4. Electrochemical impedance spectroscopy curves obtained for the tested biosensor after incubation in human serum (blank), reference human serum containing 1×10^{-12} M (0.5 mIU/mL) hCG, and human serum with 1×10^{-12} M (0.5 mIU/mL) hCG hormone addition.

4. Conclusions

In this study the oligopeptide (PPLRINRHILTR) was used to create a new electrochemical biosensor tested by the EIS method. The aforementioned oligopeptide has been used in other work for hCG detection, but in this work the EIS method was used for the first time, based on a modified gold electrode. Each modification step was confirmed by electrochemical investigations. The obtained biosensor exhibits sensitivity towards hCG in a range of concentrations from 1×10^{-12} to 1×10^{-7} M (0.5 mIU/mL – 50 000 mIU/mL) in 0.01 M PBS solution with detection limit of 1.91×10^{-14} M (0.0095 mIU/mL). The effectiveness of the investigated biosensor was also tested in human serum.

The performed comparative studies in human serum of a known concentration of hCG in human serum where the hCG was added, allow to conclude that the tested biosensor was selective at the hCG concentration of 1×10^{-12} M (0.5 mIU/mL). The use of new electrochemical biosensors may in the future contribute to the rapid determination of hCG with high sensitivity, which can have practical applications in clinical analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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