An ultrasensitive electrochemical immunosensor platform based on disposable ITO electrode modified by 3-CPTMS for early detection of parathyroid hormone

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Abstract: Parathyroid hormone (PTH) is a polypeptide containing 84 amino acids secreted by cells of the parathyroid glands. Imbalances of parathyroid levels cause medical problems such as osteoporosis, mental disorders, pancreatitis, kidney stones, cancer, and other symptoms. In this study, we aimed to design an ultrasensitive electrochemical immunosensor for PTH detection. Indium tin oxide (ITO) was used as an electrode for electrochemical impedance spectroscopy (EIS) measurements. ITO sheets were modified by using 3-cyanopropyltrimethoxysilane (3-CPTMS) self-assembled monolayers (SAMs) for immobilizing the anti-PTH antibody via covalent interactions. Cyclic voltammetry (CV) and EIS methods were applied to characterize immobilization steps. Therefore, 1% was selected for an optimal concentration of silane, 10 ng/mL was selected as an optimal concentration of anti-PTH, and 30 and 45 min were selected as optimal incubation times for anti-PTH and PTH, respectively. PTH antigen was determined in the concentration range from 0.05 fg/mL to 150 fg/mL. To detect the analytical characterization of the 3-CPTMS modified immunosensor, linear range, repeatability, reproducibility, Kramers–Kronig transform, and regeneration studies were performed. Also, the shelf life of the developed biosensor was investigated. Finally, real human serum samples were analyzed with the PTH immunosensor. The results showed that the designed biosensor system has high potential for early detection for medical treatments.

Key words: Biosensor, disposable immunosensor, ITO-PET electrode, parathyroid hormone, 3-CPTMS

1. Introduction
Parathyroid hormone (PTH) provides a balance of calcium and phosphorus ions in vertebrates. The level of PTH is regulated in the kidneys and bones by specific receptors [1]. PTH and vitamin D are known to be responsible for maintaining extracellular calcium balance. Vitamin D increases the absorption of calcium ions in the intestine. PTH is secreted in response to low circulating calcium levels. At low levels of vitamin D and high PTH levels, calcium transfers from the bones to blood. This results in increased fragility of the skeleton. Low vitamin D and high PTH levels may increase the risk of many metabolic syndromes including hyperparathyroidism, hypertension, obesity, and diabetes [2]. Depression was also associated with changes in PTH and vitamin D levels. The treatment of hyperparathyroidism has shown normalization of mood [3]. Also, low serum 25-hydroxyvitamin D (25-OH-D) [3–11] and high serum PTH [3,8,9,12,13] levels were found to be associated with obesity. Increased PTH increases calcium flow to adipocytes. Consequently, weight gain is triggered [4].

Electrochemical biosensors are based on amperometric, potentiometric, and impedimetric measurements and offer high precision, fast analysis, and unlabeled and real-time detection. Therefore, they have an important

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role in determining the target analytes for clinical diagnosis. Antibody-antigen binding in electrochemical immunosensors results in a change in the thickness of the electrode surface. Determination of the target analyte is carried out due to changes in electrochemical properties. EIS provides a precise and instantaneous measurement of these properties. In electrochemical biosensors, the interaction between the detection element and the target analyte affects the measurable electrical properties of the solution. In our previous studies, we applied a single frequency impedance technique for PTH detection in a biosensor system for the first time. Increased impedance values were reported due to the increase in PTH concentration using anti-PTH as a bioreceptor [5].

ITO is a preferred platform as an electrode for biosensor applications. It has advantages in terms of optical transparency, easy layer formation, high working potential, high stability, and easy miniaturization. For successful analyte determination, an effective modification of the ITO surface should be performed. Therefore, various methods such as physical adsorption, electrochemical deposition, electropolymerization, and silanization are used. For silanization, 3-aminopropylsiloxysilane (APTES), 3-isocyanatopropyltriethoxysilane (IPTES), 3-aminopropytrimethoxysilane, 3-glycidoxypropylmethoxysilane, 3-mercaptopropyltrimethoxysilane (3-MPTMS), and 3-glycidoxypropyltrimethanesilane are used. The silane agent allows the formation of functional groups such as -COOH, -NH₂, -SH, and -CN, which play roles in the attachment of the biodegradation element to the hydroxylated electrode surface. The silane agent forms a well-controllable self-assembling layer formation [6,7].

Clinical diagnostics require fast and reliable test systems. Instead of conventional tests with long assay times and high costs, the use of disposable electrochemical biosensors for early diagnosis of diseases has become very interesting. It is preferred because of its advantages such as sensitivity at low concentrations, selectivity, being cheap, and being easy to prepare [8]. In EIS, charge transfer resistance (Rct) is used for the measurement of target molecules. Nyquist graphs represent the EIS spectra of different layers of the modified electrode. In Nyquist graphs, the semicircular portion at high frequencies represents limited electron transfer and the linear portion represents a limited diffusion process. The diameter of the semicircle is used to calculate the Rct on the electrode surface. The most commonly used electrical circuit model for an electrochemical reaction is the Randles–Ershler electrical equivalent circuit model. This equivalent circuit model includes the ohmic resistance (Rs) of the electrolyte solution between the working electrode and the reference electrode, the Rct, the bilayer capacitance coupled with the capacitance of the complex bioactive layer, and Warburg impedance, which represents the diffusion of the redox probe from the electrode surface [9].

Miniaturization is crucial for the development of analytical tools. Both economically and practically disposable electrochemical biosensors can be used quickly and efficiently for on-site analysis. Electrochemical application of biosensors is important in many pharmaceutical analyses, including the identification of drugs, metabolites, and degradation products of different matrices. Electrochemical biosensors provide both precision and natural selectivity with a simple production process, high reproducibility, and low power requirements and costs for routine analysis for different platforms for different target analytes. The growing demand for on-site monitoring tests in biomedical, pharmaceutical, industrial, and environmental analysis has led to the development of electrochemical sensors [10]. Examples of various sensors are copper sensors [11], enzymatic sensors and immunosensors [12,13] for the detection of toxic metals, microfluidic system [14,15], and amino acids and carbohydrates [15,16] that can be used for environmental residues and food pathogens. Gold, silver, and platinum disposable electrodes are used to detect halides, cyanide, thiocyanate [17], alcohols, and arsenic [18]. These disposable sensors can be used in different electrochemical techniques in which the electrode response

1698
in an unknown solution is measured and the concentration can be determined directly from the calibration graph. Also, because of its disposable properties, the same electrode surface can be safely used for sequential analyses, since it prevents electrode poisoning from reuse [10].

Kim et al. generated electrostatically self-assembled novel nanocomposites of MoS$_2$-graphene nanosheets on top of a gold electrode and reported their biochemical amplification responses. The detection range of the designed biosensor was 0.001–10 ng/mL and the detection limit was 10 pg/mL [19]. In another study, Kim et al. reported a biosensor based on a MoS$_2$-graphene (MG) composite that can measure the PTH concentration in serum samples from patients. The interaction between PTH and MG was analyzed via an electrochemical sensing technique. Furthermore, the ALP-PTH-MG sensor exhibits a linear response towards PTH from artificial serum over a range of 1–50 pg/mL [20]. Dittmer et al. described a rapid method for the sensitive detection of proteins using actuated magnetic particle labels, which are measured with a giant magnetoresistive biosensor. For the measurement of PTH, a detection limit in the 10 pM range was obtained with a total assay time of 15 min when 300-nm particles were used [21]. PTH immunosensor studies that we designed in our previous work are available. Şimşek et al. fabricated a new electrochemical impedance-based biosensor for the analysis of PTH using self-assembled monolayer (SAMs) of mercaptohexanol and (3-aminopropyl) triethoxysilane on gold electrodes for the first time in the field. The linear PTH detection range of the presented biosensor was 10–50 pg/mL PTH [22]. Özcan et al. constructed a biosensor based on a gold electrode modified by 12-mercapto dodecanoic (12MDDA). Antiparathyroid hormone (anti-PTH) was covalently immobilized onto poly(amideamine) dendrimer (PAMAM), which was bound to a 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide/N-hydroxysuccinimide (EDC/NHS) coupled SAM structure from one of the other NH$_2$ sites. PTH was detected within a linear range of 10–60 fg/mL [23]. Özcan et al. designed a biosensor based on a gold electrode modified by mercaptohexanol (6-MHL) in another study. Antiparathyroid hormone was covalently immobilized onto a SAM by using epichlorohydrin (EPI) with ethanolamine (EA). The EPI-EA interaction represents the first use of these for the construction of biosensors in published reports. PTH was detected within a linear range of 0.1–0.6 pg/mL, and the detection limit was 0.1 fg/mL [24].

The ultrasensitive electrochemical PTH immunosensor that we design provides practical, fast, and efficient use. EIS is a highly accurate technique that performs real-time and unlabeled measurement of probe-analyte interactions. Another advantage of the developed biosensor is that the silane agent does not require any cross-linker. Compared to all other studies in the literature, the manufacturing process of the designed biosensor is very simple and it has a very wide detection range. The detection range of the biosensor was 0.05–150 fg/mL and the detection limit was 0.0269 fg/mL. It has a very wide linear detection range; therefore, its accuracy is great. High reproducibility ensures accuracy in routine analysis. Another important point is that it can be regenerated even though it is disposable.

2. Materials and methods
2.1. Chemicals and apparatus

All chemicals and ITO films were obtained from Sigma Aldrich (USA). ITO electrodes (2 mm × 20 mm) were used as working electrodes in a triple electrode system for electrochemical measurements. Additionally, Ag/AgCl as a reference electrode and platinum wire as an opposite electrode were respectively used. Ultrapure water was obtained from an Elga LC134 system (18.2 MΩ/cm). PTH and anti-PTH were procured from Sigma Aldrich and both were prepared in a phosphate buffer with pH 7 (50 mM). BSA (0.5%) was prepared in a
phosphate buffer. All electrochemical experiments were completed using a Gamry potentiostat/galvanostat (Reference 600, Gamry Instruments, Warminster, PA, USA) and electrochemical measurements were performed using a redox probe in 5 mM [Fe (CN)₆]³⁻/⁴⁻ 50 mM PBS solution containing 0.1 M KCl (pH 7.4).

2.2. Immobilization steps of biosensor

First, ITO electrodes were cleaned for 10 min each in acetone, a soap solution, and ultrapure water, respectively, with ultrasonication processes. After the cleaning procedures, for hydroxylation electrodes were left in a solution of ammonium hydroxide, hydrogen peroxide, and ultrapure water (1:1:5) for 90 min. During the procedure, a toluene-ethanol mixture (1:1) with 1% 3-CPTMS solution was prepared as a solvent medium. At the end of the incubation duration, electrodes were first passed through pure water and then toluene-ethanol (1:1) solution and left overnight in a silane solution. With covalent binding of 3-CPTMS to the antibody, self-assembled monolayers were created. Later, ITO electrodes were washed with an ethanol/toluene (1:1) mixture and then ultrapure water to remove physically adsorbed 3-CPTMS and then dried with argon gas. After the interaction of silane groups and OH groups, electrodes were incubated for 45 min in 200 μL of 10 ng/mL anti-PTH solution to immobilize the antibody on the surface. After PTH antibody incubation, electrodes were washed with ultrapure water to remove unbound antibody molecules. Finally, anti-PTH-immobilized ITO electrodes were treated with BSA (0.5%) for 60 min to block active sites. After this, electrodes were again washed with ultrapure water. After this final step, the prepared biosensor was stored at 4 °C until PTH measurements. The immobilization steps for the designed PTH immunosensor are presented in Figure 1.

![Figure 1](image-url)

**Figure 1.** Representation of immobilization steps of the PTH immunosensor on the ITO electrode surface.

2.3. Electrochemical measurements

The electrodes with completed immobilization steps were treated with standard PTH solutions prepared at different concentrations. For each PTH concentration, EIS and CV measurements were taken. Electrochemical
measurements were performed with a redox probe. The potential applied for CV measurements was from –0.5 V to 5 V, with step size of 10 mV and scanning rate of 100 mV/s. For impedance studies, the applied formal potential was 0 V with 5 mV alternative current. Impedance measurements were completed in the frequency interval from 50,000 to 0.05 Hz. For square wave measurements, the potential interval was 0 V to 1.4 V, pulse size 25 mV, and step size 5 mV. All measurements were performed in 20 mL of 5 mM ferricyanide/ferrocyanide redox probe solution.

2.4. Optimization steps of biosensor

In order to obtain a sensitive and reproducible biosensor, parameters like silane concentration, anti-PTH concentration and incubation duration, and PTH incubation duration were optimized.

To determine the effect of silane concentration on the response of the designed immunosensor, the biosensor was incubated in three different silane concentrations (0.5%, 1%, 1.5%) and the capacity to determine PTH in solutions with different concentrations was tested. Based on the impedance curves, the calibration graphs for biosensors prepared with different silane concentrations were drawn and the optimum silane concentration was chosen.

The biosensors were incubated in three different anti-PTH solutions (5 ng/mL, 10 ng/mL, 20 ng/mL) to be able to test the effect of anti-PTH concentration on immunosensor response and the capacity to determine PTH in solutions with different concentrations was identified. The calibration graphs were drawn from the impedance curves for the biosensor prepared with different anti-PTH concentrations and the optimum anti-PTH concentration was determined.

After anti-PTH concentration, for optimization of the incubation duration, electrodes were left in anti-PTH solution for different durations (30 min, 45 min, 60 min) and the prepared biosensors were used to determine different concentrations of PTH solution. Based on the obtained EIS curves, the calibration graphs for different incubation durations were drawn and optimum anti-PTH incubation durations were chosen.

Electrodes were left in PTH for different periods (30 min, 45 min, 60 min) in order to investigate the effect of PTH incubation duration on the response of the immunosensor. Calibration graphs were drawn based on the obtained impedance curves and optimum PTH incubation duration was chosen.

2.5. Characterization study of the constructed biosensor

In this stage, the biosensors prepared under the optimum conditions were incubated in increasing PTH concentrations and EIS and CV measurements were taken. The variation in Rct graph was drawn linked to increasing PTH concentration. Impedance calculations were made using an equivalent circuit model.

The Kramers–Konig transformation was used to determine whether the impedance spectrum of the PTH biosensor was affected by external factors or not and whether deviation occurred or not.

One of the primary features required for a sensitive and linear immunosensor is repeatability. For this purpose, 20 different biosensors prepared under the same conditions were incubated in the same concentration of PTH (50 fg/mL) and EIS measurements were taken. The obtained results were statistically analyzed. Standard deviation, mean values, and variation coefficient calculations were performed.

For reproducibility study of the PTH biosensor, eight anti-PTH immobilized biosensors prepared under the same conditions, but at different times, were incubated in increasing PTH concentrations and the responses were compared. This procedure was repeated ten times.
Electrodes prepared under optimum conditions and stored at 4 °C were incubated in the same concentration of PTH (50 fg/mL) at weekly intervals for the storage test and EIS measurements were completed.

In the regeneration step, the capacity of the biosensor prepared under optimum conditions and treated with PTH to have the antibody-antigen interaction disrupted and then form the antibody-antigen interaction again when treated with antigen was tested. After PTH incubation, the electrode was left in 0.1% HCl acid for 5 min. Then the electrode was washed with ultrapure water and treated with PTH once more. Each step was monitored with EIS and the procedure was repeated until the biosensor activity was largely lost.

The characteristic features of the designed biosensor were tested with square wave voltammetry (SWV). Electrodes prepared under optimum conditions were treated with PTH in the linear detection interval and SWV was performed. The graph of the observed peak current against increasing PTH concentration was drawn.

With the aim of investigating the response of the designed PTH biosensor to real serum samples, five different human serum samples were studied. The PTH amount in serum was measured with the standard addition method. In human serum, PTH levels above 55 pg/mL are accepted as high and below 10 pg/mL are accepted as low [25]. Serum samples were diluted to a concentration of 50 fg/mL (dilution factor: 1000 times) in the linear interval of the designed biosensor. The PTH concentrations used for standard addition were 10 fg/mL and 50 fg/mL. The impedance data obtained from measurements were calculated using the equation on the calibration graph.

3. Results and discussion

Biosensors are promising analytical tools that can be applied during clinical diagnosis. They play an important role in measuring specific compounds in biological fluids like blood and plasma. Electrochemical biosensors are simple, rapid, and cheap devices. They have very sensitive detection limits to monitor variable hormone levels in healthy and patient sera. Thus, they can be safely chosen for clinical diagnosis. What makes electrochemical biosensors applicable as point-of-care diagnostic kits is that they can be miniaturized to very small sizes. Glucose biosensors and home pregnancy and ovulation tests can be given as examples. Limitations to the commercialization of most biosensors are their expense, difficult regeneration stages, and lack of good repeatability. As a result, to transform this technology into commercial products the investment of more time and money is required [10].

EIS is a sensitive technique allowing real-time and label-free measurement of probe-analyte interactions. In this study, the aim was to design a sensitive electrochemical immunosensor based on ITO for PTH determination. EIS and CV techniques were used to determine the amount of PTH antigen on the electrode surface of the biosensor. All electrochemical measurements used ITO as a working electrode in a triple electrode system, with silver chloride (Ag/AgCl) as a reference electrode and platinum wire as a counter electrode. In addition to the EIS technique, SWV, Kramers–Konig, and regeneration techniques were used for characterization of the electrode surface. Optimum operating conditions were tested with calibration curves obtained during optimization experiments and the reproducibility and repeatability of the PTH biosensor were also tested. The designed immunosensor had a wide linear detection interval (0.05–150 fg/mL) and low limit of detection (LOD) (0.0269 fg/mL). Another important point is that although the developed immunosensor is for single use, sequential regeneration is possible.

Additionally, results obtained from real human serum samples showed that the developed immunosensor can be successfully used for the determination of PTH. The test duration requires at least 1 h to be completed and this is very short compared to traditional ELISA experiments, which take longer. This largely simplifies the sample preparation procedure and all test procedures.
3.1. Immobilization steps of biosensor

The EIS curves and CV voltammograms for the immobilization steps of the biosensor designed for PTH determination are shown in Figure 2. The goodness of fit values of the measurements from the immobilization steps in Figure 2 were calculated and presented as a table (Table 1). The results indicate that immobilization takes place.

![Figure 2. A) EIS and Randles equivalent circuit (Rs: ohmic resistance, Rct: charge transfer resistance, CPE: constant phase element, W: Warburg impedance); B) CV responses of immobilization steps of the PTH immunosensor.](https://example.com/figure2.png)

**Table 1.** The goodness of fit values of the measurements that created the immobilization steps.

<table>
<thead>
<tr>
<th>Biosensor surfaces</th>
<th>Goodness of fit values</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITO/OH</td>
<td>0.84</td>
</tr>
<tr>
<td>ITO/OH/3-CPTMS</td>
<td>1.05</td>
</tr>
<tr>
<td>ITO/OH/3-CPTMS/Anti-PTH</td>
<td>1.21</td>
</tr>
<tr>
<td>ITO/OH/3-CPTMS/Anti-PTH/BSA</td>
<td>1.33</td>
</tr>
<tr>
<td>ITO/OH/3-CPTMS/Anti-PTH/BSA/PTH</td>
<td>1.54</td>
</tr>
</tbody>
</table>
EIS is a very effective method used to study many chemical and physical processes. Electrochemical biosensors offer high sensitivity, rapid analysis, and label-free and real-time operation. As a result, they play an important role in determining the target analyte for clinical diagnosis. In electrochemical immunosensors, antibody-antigen binding causes changes in the thickness on the electrode surface. The determination of the target analyte is completed linked to changes in the electrochemical properties. EIS ensures the sensitive and instant measurement of changes in these properties. When the EIS curves are investigated, the Rct value of the hydroxylated electrode surface falls and the radius of the Nyquist diagram appears to be very low linked to this (Figure 2A). When the electrode surface is modified with 3-CPTMS silane agent, there is an increase observed in the Rct value. Covalent bonding of the silane group (-O-Si-O-) on the 3-CPTMS silane agent and the hydroxyl group (-OH) on the ITO surface forms self-assembled monolayers on the electrode surface. As a result of interaction of cyano groups from the silane agent with the negative charge of the redox probe, the redox probe makes diffusion to the electrode surface difficult. Accordingly, in the EIS spectrum, the 3-CPTMS signal increases compared to the -OH signal. In the next step of treatment with anti-PTH, cyano groups of the silane agent and amine groups in the anti-PTH structure enter covalent interactions and form a barrier on the electrode surface leading to an increase in Rct. BSA was used as a blocking agent with the aim of closing active sites that had not interacted with amine groups. The electrode surface was then insulating and Rct increased. Finally, for PTH measurement, the electrode surface was incubated in the antigen and weak interaction occurred between the antibody and antigen; in this step, the Rct value was observed to increase.

For all immobilization steps, in addition to EIS measurements, CV measurements were performed to evaluate the variations on the electrode surface. CV is used for processes where cyclic electron transfer occurs. The CV voltammograms for all immobilization steps of the biosensor developed for PTH detection are given (Figure 2B). With the increasing insulation of the electrode surface, peak currents were observed to largely reduce.

3.2. Optimization steps of biosensor

Optimization involved the optimization of 3-CPTMS and anti-PTH concentrations and anti-PTH and PTH antibody incubation durations. To identify the optimum 3-CPTMS concentration for the first optimization step of the designed biosensor, three different values were chosen (0.5%, 1%, 1.5%). Intensive coating of the electrode surface with the silane agent will reveal the possibility of retaining large amounts of protein on the surface. This may cause a steric barrier to the binding of the antigen, leading to a decrease in the performance of the electrode. Conversely, the sensitivity of the biosensor will decrease if sufficient immobilization of the antibody is not achieved. The EIS signals obtained with 0.5% concentration appeared to be lowest (Figure 3A). This shows that this concentration was not sufficient for formation of a successful immobilization layer on the electrode surface. Similarly, for the 1.5% concentration value, the EIS signals were observed to be lower compared to the 1% value. The reason for this is that an inefficient immobilization layer formed linked to intense binding at high concentrations. Finally, the optimum value of 3-CPTMS concentration was chosen as 1%.

Another optimization step was completed with anti-PTH. Three different anti-PTH concentrations were chosen (5 ng/mL, 10 ng/mL, 20 ng/mL). The highest response appeared to be obtained with anti-PTH at 10 ng/mL concentration (Figure 3B). The value for 5 ng/mL was insufficient and the value for 20 ng/mL may be interpreted as a low signal due to formation of density on the layer. In conclusion, the optimum anti-PTH concentration was chosen as 10 ng/mL. After determining the optimum concentration of anti-PTH, the incubation duration was optimized. With this aim, optimization of the anti-PTH incubation duration
Figure 3. Optimization linear graphs of the PTH immunosensor: A) standard curves obtained by different 3-CPTMS concentrations; B) standard curves obtained by different anti-PTH concentrations; C) standard curves obtained by different anti-PTH incubation times; D) standard curves obtained by different PTH incubation times.

was completed with three different durations (30 min, 45 min, 60 min). The signals appeared to reduce with increasing duration (Figure 3C). As a result, an incubation duration of 30 min was chosen as the optimum value for short duration and high response. In the final step, the PTH antigen incubation duration was optimized. Three different incubation durations (30 min, 45 min, 60 min) were determined. It can be considered that 30 min of antibody-antigen interaction is not a sufficient duration (Figure 3D). However, 60 min reduced the PTH binding capacity of the antibody and the response was observed to fall. The optimum value of 45 min of incubation duration was seen as appropriate.

3.3. Characterization steps of biosensor

The EIS spectra and cyclic voltammograms for the designed immunosensor after completing all immobilization steps and optimization processes are given with increasing PTH concentrations (Figures 4A and 4B). As the Rct values increase due to increasing antigen concentrations, there is a reduction observed in peak current for cyclic voltammetry. The experimental data for the Kramers–Konig transformation appear to overlap with the virtual calculations for the components (Figure 4C). The transformation is used to determine whether the impedance spectrum of the developed biosensor system is affected by external factors and deviations or not [5]. In Figure 5, the calibration graph for the biosensor is given. As can be seen, the designed biosensor has a wide linear detection interval (0.05–150 fg/mL). Additionally, LOD and limit of quantification (LOQ) values were calculated as 0.02690 fg/mL and 0.08966 fg/mL, respectively. When calculating LOD and LOQ values, the “k·S (standard deviation)/m (slope of curve)” equation was used. The k value was accepted as 3 for LOD and 10 for LOQ [26].
Figure 4. A) Determination of increasing concentrations of PTH EIS spectra; B) determination of increasing concentrations of PTH CV voltammograms; C) Kramers–Kronig transformation of the PTH immunosensor.

Figure 5. Calibration curve of designed PTH biosensor.

Repeatability studies are an important parameter showing the stability of the designed biosensor. The same concentration is used to test the capability of the biosensor for consecutive measurements. Twenty different electrodes were prepared under the same conditions and electrodes were incubated in 50 fg/mL PTH concentration for impedance measurements. Calculated according to the calibration equation of the impedance
measurements, the mean value, standard deviation, and immunosensor variation coefficient values were obtained as 50.277 fg/mL, 1.306 fg/mL, and 2.598%, respectively. Based on this, it can be concluded that the designed biosensor has high repeatability.

Overlapping linear graphs showing reproducibility of the PTH biosensor are given in Figure 6A. For reproducibility, PTH biosensors were prepared under the same conditions and impedance measurements were completed in the linear detection interval (0.5–150 fg/mL). This procedure was repeated ten times. Each independent reproducibility study proves the stability of the designed PTH biosensor.

![Reproducibility Study](image)

**Figure 6.** A) Reproducibility study; B) regeneration study; C) storage life; D) square wave voltammetry of the designed PTH immunosensor.

Though the designed biosensor is single-use, regeneration procedures were performed and it was observed that the biosensor preserved high rates of activity (Figure 6B). This regeneration procedure involved treatment of the ITO electrode, prepared under the same conditions, with HCl solution (10 mM, 5 min) to disrupt the weak interaction between antibody and antigen after 45 min of incubation in PTH antigen (50 fg/mL). After each acid procedure, the surface was again incubated in PTH antigen (50 fg/mL) and impedance measurements were taken. The designed PTH immunosensor had this regeneration procedure repeated 14 times. Though disposable, the ITO working electrode was observed to preserve it properties with repeated use.

The storage life of the PTH immunosensor was tested (Figure 6C). It is expected that an ideal biosensor will have a long storage life. As a result, storage measurements were completed over 10 weeks. Electrodes prepared under the same conditions were stored in the dark at 4 °C and EIS measurements were taken each
week after electrodes were incubated in 50 fg/mL PTH. The designed biosensor lost only 10.51% of its relative activity after 10 weeks.

The response of the designed biosensor to SWV was observed. SWV rapidly identifies biomarkers with high sensitivity. The calibration graph observed in Figure 6D shows the peak current values obtained in the 0.5 to 150 fg/mL detection interval. Peak current values show a reduction as the PTH concentration increases.

3.4. Applicability of PTH biosensor to real serum samples

The response of the designed biosensor to real serum samples was tested. The relative standard deviation and recovery values are calculated in Table 2. According to the obtained results, the error value is low and the designed biosensor can be said to have high potential for PTH determination in the clinical field.

Table 2. Application of PTH-based biosensor to real serum samples.

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<th>Serum sample number</th>
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<th>Standard added conc. (fg/mL)</th>
<th>Measured conc. (fg/mL, n = 3)</th>
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<th>Recovery (%)</th>
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</table>

4. Conclusion

In this study, an ultrasensitive, stable, and quite repeatable electrochemical PTH immunosensor with a disposable, cost-effective, and practical ITO PET working electrode has been successfully constructed. After the PTH immunosensor was fabricated, a great number of optimization and characterization studies were carried out. The designed biosensor has a wide linear detection range (0.05–150 fg/mL) and low LOD (0.02690 fg/mL) and LOQ (0.08966 fg/mL). It can perform accurate measurements at femtogram levels. Its high reproducibility indicates the stability of the designed biosensor. Although disposable, it was observed that the ITO working electrode maintained its activity even in repeated studies. Finally, based on the test results for real human serum samples, it can be concluded that the designed biosensor system has very high potential for early detection of medical treatments.

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