



A direct and simple immobilization route for immunosensors by CNBr activation for covalent attachment of anti-leptin: obesity diagnosis point of view

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Abstract

Leptin is a peptide hormone produced in adipose tissue that works as an antiobesity hormone by balancing energy intake and expenditure. We aimed to develop an ultrasensitive electrochemical immunosensor based on a novel immobilization technique for the early detection of leptin-related diseases in this work. Although several methods for immobilizing antibodies to the biosensor recognition element are known, it is necessary to utilize novel, cost-effective, and less complicated immobilization procedures. When compared with currently utilized immobilization techniques for leptin measurement, this novel method is more efficient, easy to prepare, and sensitive, with a broad detection range. Indium tin oxide-coated polyethylene terephthalate (ITO-PET) sheets were used as the working electrode. ITO-PET sheets were modified using cyanogen bromide (CNBr) to immobilize the anti-leptin antibody through covalent interactions. Each stage of the proposed biosensors was characterized by electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) methods, and extensive characterization studies were carried out. The designed biosensor has a wide linear detection range (0.05–100 pg/mL), low limits of detection (LOD) (0.0086 pg/mL) and quantification (LOQ) (0.0287 pg/mL). It was concluded that although it is disposable, the ITO-PET working electrode retains its activity even in repeated studies. In addition, the new immobilization procedure provided by CNBr for the designed biosensor fabrication can be effectively used in other biosensing applications.

Keywords ITO-PET electrode · Leptin · Cyanogen bromide · Electrochemical impedance spectroscopy · Cyclic voltammetry

Introduction

Biomarkers are used to monitor disease progression, risk assessment, clinical diagnosis, and various other processes. Biomarkers used include enzymes, antibodies, nucleic acids, whole cells, receptors, tissues, organelles, and more (Salek-Maghsoudi et al. 2018). These molecules, which indicate an abnormal condition or disease in body fluids or tissues, are of great importance to significantly reduce disease-related morbidity and mortality in early diagnosis. Leptin was used as a biomarker in this study's new electrochemical biosensor system.

Leptin, a 167 amino acid-containing peptide hormone produced by white adipose tissue, was discovered in 1994 by the Jeffrey Friedman laboratory. The primary function of leptin is to protect food intake and energy consumption in the human body, so leptin is considered a satiety hormone (Perumal et al. 2019). Plasma leptin concentration is proportional to body adiposity and significantly increased in obese individuals. High leptin concentrations are directly related to obesity and metabolic diseases, such as insulin resistance, type 2 diabetes, and cardiovascular diseases. Leptin is available in two forms: free in blood and protein bound. The free form is thought to be responsible for the activity of leptin. Studies have shown that most of the leptin in serum is in free form in obese individuals. Therefore, determining the increase in free leptin form in obese people is seen as one of the pieces of evidence supporting the hypothesis that the main problem in the development of obesity is not leptin deficiency but leptin resistance (Benbaibèche et al. 2020; Bosy-Westphal and Müller 2021; Dey et al. 2021).

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However, further studies have shown that leptin is associated with regulation of gastrointestinal functions, the realization of angiogenesis, regulation of sympathetic nervous system activation, determination of bone density, thermogenesis and brain development, cell viability, proliferation, and migration, and immunity. Continuous leptin monitoring can also explain the progression of clinical pathologies (Ghadge and Khaire 2019). The current routine method for leptin testing is ELISA, but it is characterized by low sensitivity, complex procedures, and uneven results (Benbaibech et al. 2020; Eleftheriou et al. 2004; Zhang et al. 2018). In addition, the traditional diagnosis of the pathophysiology of obesity is based on body mass index (BMI) due to excess fat storage. However, quantitative and qualitative analyses of lean and adipose tissue fractions in body composition are not considered sufficient for a comprehensive understanding of obesity-related health risks (Bosy-Westphal and Müller 2021). Instead of standard tests with long assay times and high costs, disposable electrochemical biosensors for the early diagnosis of diseases have recently become quite interesting. Electrochemical techniques are preferred due to their selectivity, cost-effectiveness, ease of preparation, and sensitivity at low concentrations (Yılmaz et al. 2019). Electrochemical biosensors are simple to fabricate, have a high amount of repeatability, low power consumption, relatively low cost, precision, and natural selectivity for a wide variety of target analytes. Electrochemical sensors were developed in response to the growing demand for onsite monitoring tests in biomedical, pharmaceutical, industrial, and environmental analyses (Bahadır and Sezgentürk 2015). Although antibody-based methods are extensively utilized in research, various recognition components, including aptamers and molecularly imprinted polymers, have been used in leptin biosensors recently. Numerous biosensor methods for the detection of leptin in a variety of samples have been published recently. Zhang et al. developed an electrochemical biosensor for leptin detection using a glassy carbon electrode (GCE) covered with single-walled carbon nanotubes (SWCNTs) distributed in chitosan (CS) solution. The biosensor has a detection limit of 5 pg/mL and an extensive linear range (0–1000 ng/mL) (Zhang et al. 2018). Cai et al. developed a porous graphene-functional black phosphorus (PG-BP) composite immunosensor to determine the *in vivo* concentration of leptin, a biomarker for nonalcoholic fatty liver disease (NAFLD). The PG-BP modified electrodes were constructed using gold nanoparticles, cysteamine, and glutaraldehyde. Under ideal circumstances, the suggested immunosensor has a detection limit of 0.036 pg/mL and a linear range of 0.150–2500 pg/mL (Cai et al. 2019). Mihailescu et al. developed two novel biomimetic electrochemical sensors to detect adiponectin and leptin by combining molecularly imprinted polymers (MIPs) with gold working electrodes (GWEs). Adiponectin and leptin have

detection limits of 0.25 g/mL and 0.110 ng/mL, respectively (Mihailescu et al. 2020). Sung and colleagues developed an electrochemical biosensor to detect leptin using o-Phenylenediamine (oPD) on screen-printed gold electrodes (SPGEs). The developed biosensor has a detection limit of 0.033 ng/mL for leptin concentrations ranging from 0.1 to 20 ng/mL (Sung and Heo 2020). Cavallo et al. created aptasensors that detect proteins by polymerase chain reaction utilizing short DNA aptamers (PCR). Human leptin was used as the target protein in this technique. The suggested aptasensor has a detection limit of 100 pg/mL (Cavallo et al. 2021).

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 electrolyte solution's ohmic resistance (R_s) between the working electrode and the reference electrode, the charge transfer resistance (R_{ct}), the double-layer capacitance connected by the capacitance complex bioactive layer, and Warburg. Warburg impedance represents the diffusion of the redox probe from the electrode surface (Törer et al. 2018). Thus, almost any process that changes the conductivity of a system can be recognized by EIS. EIS may also be used to determine the variable current densities, charge transfer resistances, double layer capacitances, and other critical characteristics of a studied electrochemical system. EIS can also be used to estimate physical parameters such as surface roughness and porosity of an electrode. Recent research has focused on developing electrode designs, miniaturization, efficient electron transfer, nanomaterials, the development of the immobilization method (Campuzano et al. 2021; Gupta et al. 2021; Peng et al. 2020; Randviir and Banks 2013; Wang et al. 2020).

The selection of the appropriate immobilization method in biosensor design is essential for the immobilization process. Enzymatic activity, the efficiency of protein use, regeneration characteristics, cost of the process, the toxicity of immobilization reagents are factors to be considered (Chiou and Wu 2004). Biological materials (enzymes, antibodies, and cells) can be attached to the matrix with irreversible chemical bonds or reversible physical adsorption. Covalent immobilization involves a robust covalent bond formation between the biological material and the carrier matrix. Among the various materials used for immobilization, polymers represent an excellent class of support material (Azarikia et al. 2015).

Two conventional methods activate the matrix by adding a reactive function to the matrix and modifying the polymer backbone to produce an activated group (Guisan 2013). Matrix selection plays a critical role in immobilization depending on cost, binding capacity, hydrophilicity, structural stiffness, and durability during various applications. ITO-PET is a preferred platform as an electrode in biosensor applications. Its benefits include excellent stability, ease

of layer creation, a wide range of application possibilities, and ease of miniaturizing. It is favored for use in various processes, including physical adsorption, electrochemical deposition, and electropolymerization (Özcan et al. 2014). Cyanogen bromide, carbodiimide, and triazine can all be used to activate matrices. The methods used to activate polysaccharides are based on the reaction between cyanogenic halides and the hydroxyls of the polysaccharide matrix. Inert support materials such as cellulose, sepharose, and Sephadex contain CNBr-activated glycol bound to enzymes. The electrophilic cyanide produced by CNBr is attacked by nucleophiles such as amines, alcohols and thiols. Organic chemists use this feature of the reagent. CNBr has also been applied in molecular biology and DNA duplexes as a binding agent for phosphoramidite or pyrophosphate internucleotide bonds (Ghadge and Khaire 2019; Kumar 2005). CNBr has lately received interest for its potential use in oligonucleotide probe binding, enzyme immobilization, and bioconjugation (García-García et al. 2020; Geissler et al. 2020; Moradi et al. 2021).

There is a lack of research on identifying disorders that leptin levels can monitor and developing a low-cost, simple-to-prepare testing gadget with higher sensitivity. CNBr was employed successfully and realistically without using crosslinkers or markers in this antibody–antigen-specific interaction-based method. Additionally, CNBr significantly enhanced the system's stability and sensitivity. The developed leptin biosensor is distinguished by its exceedingly low detection limit (0.0086 pg/mL). It is predicted that the electrochemical biosensor constructed would evolve into a design that is easily applicable to various target biomarkers. Moreover, this method is well suited for immobilizing enzymes, antibodies, peptide hormones, and similar biologically active molecules.

Materials and methods

Chemicals and materials

All chemicals and ITO films were purchased from Sigma Aldrich (USA). For electrochemical measurements, ITO electrodes (2 mm × 20 mm) were used as working electrodes in the triple electrode system. Also, Ag/AgCl was used as the reference electrode, and platinum wire was used as the counter electrode. Ultrapure water was obtained from the Elga LC134 system (18.2 MΩ/cm). Leptin and anti-leptin were purchased from Sigma Aldrich, and both were prepared with pH 8.0 Tris–HCl buffer (20 mM). 0.5% BSA was prepared with phosphate buffer (pH 7). All electrochemical experiments were carried out using Gamry potentiostat/galvanostat (Reference 600, Gamry Instruments, Warminster, PA, USA), and electrochemical measurements were 5 mM

[Fe(CN)₆]^{3−/4−} 50 mM PBS containing 0.1 M KCl used as a redox probe solution (pH 7.4). JEOL JSM-7100F brand Scanning Electron Microscope (SEM) available in Çanakkale Onsekiz Mart University (ÇOBILTUM) Scientific and Technological Research Center was used to examine the morphological changes that occur on the surface during the immobilization steps of the biosensor.

Electrochemical measurements

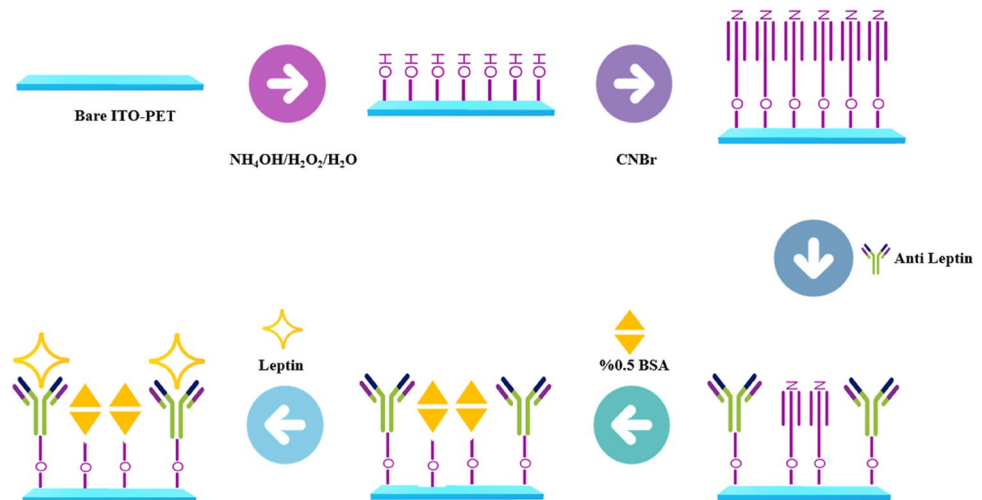
After the immobilization steps, the electrodes were treated with standard leptin solutions prepared in different concentrations. Electrochemical impedance spectroscopy and cyclic voltammetry measurements were taken to investigate surface behavior at each optimization step and different Leptin concentrations. Electrochemical measurements were taken in the redox probe. The potential applied for CV measurements was chosen between − 0.5 V and 5 V, the step size is 10 mV, and the scan speed is 100 mV/s. In alternating current, the formal potential applied in impedance studies was 0 V and 5 mV. Impedance measurements were completed in the frequency interval from 50,000 to 0.05 Hz.

Methods

Design of the biosensor

ITO electrodes were cleaned with acetone, soap solution, and ultrapure water for 10 min, respectively. After cleaning, the electrodes were kept in a solution containing ammonium hydroxide, hydrogen peroxide, and ultrapure water (1:1:5) for 90 min to form hydroxyl groups on the surface. On the other hand, cyanogen bromide was weighed at a 2 mg/ml concentration and dissolved with acetonitrile. The pH of the CNBr solution was adjusted to be essential (pH ~ 12.0) with NaOH (2 M) solution. A fresh CNBr solution was prepared for each study. At the end of the incubation period, the electrodes were soaked in the CNBr solution for 45 min. Optimization studies fixed the specified values. Then the ITO electrodes were washed with acetonitrile and ultrapure water, respectively, to remove physically adsorbed CNBr molecules and then dried in argon gas. Cyanate groups, which are expected to occur after the interaction of cyano groups with OH groups on the electrode surface, formed the surface suitable for the immobilization of antibodies. To immobilize antibodies, each electrode was incubated in 200 µL of 74 ng/mL anti-leptin solution for 45 min. After incubation with the leptin antibody, the electrodes were washed with ultrapure water to remove unbound antibody molecules. Finally, the electrodes were treated with BSA (0.5%) for 60 min to prevent nonspecific binding on the electrode surface and washed with ultrapure water. The biosensor prepared with this last step was stored at + 4 °C until Leptin

Scheme 1 The immobilization steps for the designed leptin immunosensor



antigen measurements were done. The immobilization steps for the designed Leptin immunosensor were presented in Scheme 1.

Optimization stages of the biosensor

Optimizing parameters such as CNBr, anti-leptin, leptin concentrations, and incubation time are crucial for producing an accurate and repeatable biosensor. The electrodes were incubated with four different concentrations of CNBr (0.2, 1, 2, and 4 mg/mL), and the modified electrode's capacity to detect various leptin solutions was determined. Using impedance curves, calibration graphs for biosensors made with various CNBr concentrations were constructed, and the ideal value was determined. Then, three different values, 30, 45, and 60 min, were tried for the incubation duration of CNBr, and the optimum time was determined. The same steps were performed for anti-leptin concentration (37, 74, and 111 ng/mL), anti-leptin incubation time (15, 30, 60, and 75 min), and leptin incubation time (30, 45, and 60 min).

Characterization stages of the biosensor

We incubated biosensors produced under optimal parameters with increasing leptin concentrations and performed EIS and CV measurements. The equivalent circuit model was used to determine the R_{ct} value, which changes with the concentration, and a calibration graph for the constructed biosensor was generated. Simultaneously, the Kramers–Kronig transformation was performed to determine if the impedance spectrum of the leptin biosensor deviated. Repeatability is a critical characteristic of a sensitive and linear immunosensor. The repeatability of the biosensor was determined by incubating 20 different biosensors with leptin at the same concentration (50 pg/mL) and performing EIS measurements. In the reproducibility investigation, 10 distinct immunosensors

made under identical circumstances were tested by measuring varying amounts of leptin antigens. Measurements were performed in three replicates, and the statistical evaluation of the study was made by calculating the relative standard deviation (RSD) of the collected results. Moreover average value, and coefficient of variation calculations were performed. Also, leptin biosensors were prepared at the same conditions but at different times and by different people, and the responses received were compared. This process was repeated ten times to test the reproducibility of the biosensor. Electrodes prepared simultaneously under optimum conditions and stored at +4° C were incubated with leptin antigen at the same concentration (50 pg/mL) at weekly intervals, and EIS measurements were performed to test the storage life. Although the electrodes are disposable, the regeneration ability was tested. The electrode was kept in 0.1% HCl for 5 min after the leptin incubation. Then it was washed with ultrapure water and treated with leptin again. EIS followed each step, and these processes were repeated until the biosensor had lost its activity to a great extent. Also, SFI (Single Frequency Impedance) technique was used in this study to monitor antigen and antibody interactions. For the measurement, the fixed frequency value was selected as 10 Hz from the Bode graph. SFI measurement was taken in the Tris–HCl buffer (pH 8.0).

Applicability of leptin biosensor to real serum samples

Five different human serums were studied to examine the designed leptin biosensor's response to real serum samples. The amount of leptin in the serum was measured by the standard addition method. The average leptin level in the blood can be considered 8.44 ± 7.69 ng/mL for an adult male and 24.58 ± 18.98 ng/mL for an adult woman (Ma et al. 2009). Samples were diluted a thousand times to evaluate serum samples in the linear range of the designed biosensor.

Leptin concentrations used for standard addition are 10 pg/mL and 50 pg/mL. The impedance data from the measurements were calculated using the equation obtained in the calibration graph.

Imaging of surface morphology of leptin biosensor with a scanning electron microscope (SEM)

SEM visualized the morphological changes occurring on the electrode surface at each immobilization stage of the designed Leptin biosensor.

Results

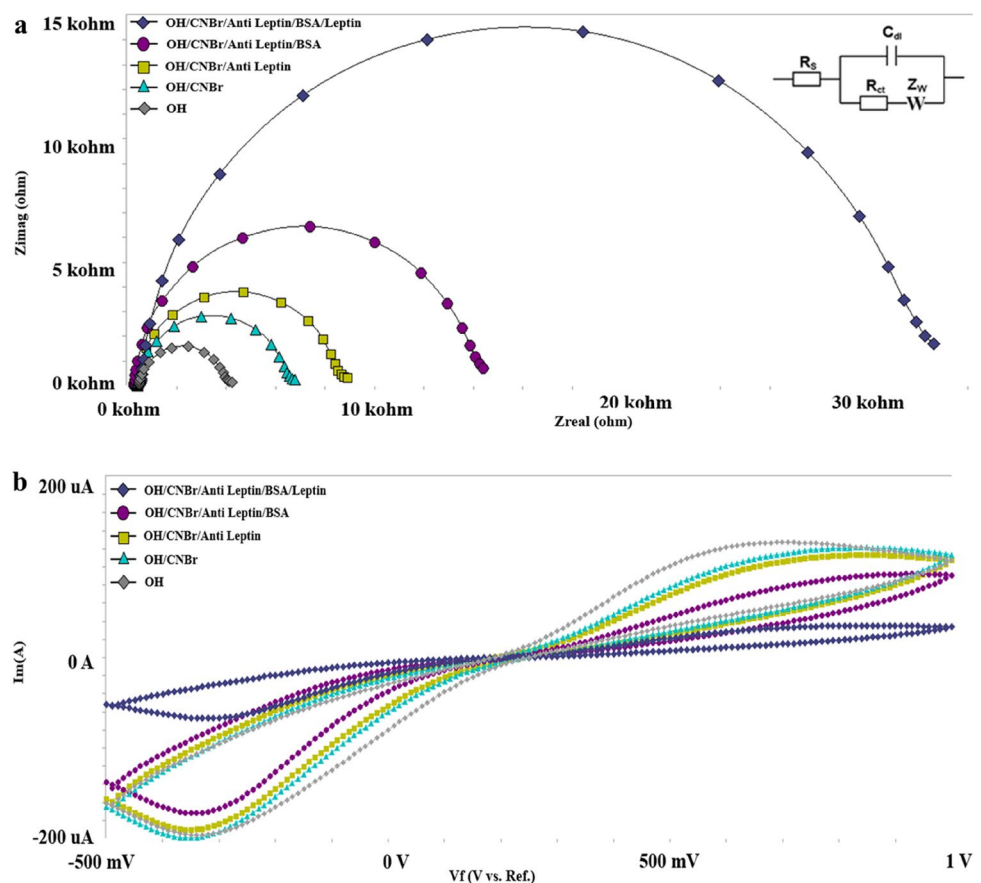
The immobilization stages of the biosensor

EIS curves and CV voltammograms of the immobilization stages of the biosensor designed for leptin determination are shown in Fig. 1. Depending on the modifications carried out, the thickness of the electrode surface and its conductivity properties change. In this way, the determination of the target analyte can be performed precisely. Since the ITO-PET electrode is a semiconductor, it has very high resistance values, so we cannot measure the bare surface. By covalently

bonding hydroxyl groups (-OH) to the ITO-PET surface, self-assembled monolayers formed on the electrode surface. In Fig. 1a, the R_{ct} value of the hydroxylated electrode surface has decreased. Based on this, it can be said that the hydroxylation process is successful. The electrode surface modification with CNBr showed an increase in R_{ct} value as compared to the previous EIS signals. In S1, the formation of a covalent bond between the hydroxyl groups and the cyano (CN^-) group at basic pH was schematized. As a result, the R_{ct} value increased. The negatively charged cyano groups and the redox probe repel each other, and an extra layer forms on the electrode surface simultaneously. The next step involves forming a covalent connection between the electrophilic cyanide groups and the nucleophilic amine groups via anti-leptin incubation.

BSA (0.5%) was used as a blocking agent to block active ends that did not interact with amine groups. As the electrode surface becomes insulating, the R_{ct} increased. Finally, antibody–antigen interactions occurred on the electrode surfaces incubated in the leptin in increasing concentrations. It was observed that the value of R_{ct} increased in increasing leptin concentrations. In addition to EIS measurement, CV measurement was taken to support the variations on the electrode surface in all immobilization steps. CV voltammograms were shown in Fig. 1b for each immobilization phase

Fig. 1 a EIS curves b CV voltammograms of the immobilization stages of the biosensor designed for leptin determination



of the designed biosensor. Peak currents were reported to be significantly decreased due to the electrode surface's higher insulating characteristics.

Optimization stages of the biosensor

The % activity values of the EIS signals received from the biosensor system were compared for each parameter. The biosensor device that produces the greatest EIS signals is the most efficient for determining leptin concentrations.

The biosensor's initial optimization phase is to optimize the CNBr concentration. The optimal CNBr concentration was determined using 0.2, 1, 2, and 4 mg/mL values. When S2 is analyzed, it is seen that the EIS signals obtained at a concentration of 0.2 mg/mL are the lowest. This concentration is insufficient to create an effective immobilization layer on the electrode surface. However, EIS signals at 4 mg/mL were found to be weaker than those at 2 mg/mL. A weak immobilization layer might form due to aggregation produced by high concentrations.

Consequently, it was discovered that a CNBr concentration of 2 mg/mL was optimal. Following that, incubation times of 30, 45, and 60 min were attempted for CNBr. Similarly, it was determined that when the period for determining leptin was shortened or surpassed, the biosensor system's effectiveness dropped. 45 min was determined to be the optimal incubation period. Anti-leptin concentration optimization has been attempted at 37, 74, and 111 ng/mL concentrations. As shown in S2 c, the percentage activity value increased as the antibody concentration increased, and similar results were achieved at high leptin concentrations of 74 and 111 ng/mL. On this basis, a 74 ng/mL concentration was determined as the optimal number for achieving an efficient system while using less material. Then, values of 15, 30, 60, and 75 min were tried for the incubation duration with the antibody. Although increasing signals were obtained in increasing periods for 15, 30, and 60 min, the leptin determination for 75 min was the lowest. However, since close signals were obtained when the 30 and 60 min incubation times were evaluated, 30 min was chosen for practical. As the last step, the incubation time of the leptin antigen has been optimized. Incubation times of 30, 45, and 60 min were tested. When S2 e is examined, it can be thought that 30 min is not enough time for antibody–antigen interaction. On the other hand, for 60 min, the leptin binding capacity of the antibody decreased according to the 45 min incubation time. The incubation time of 45 min was considered as the optimum value.

Characterization stages of the biosensor

When Figs. 2a, b are examined, the EIS spectrum and CV voltammograms were obtained in the leptin determination

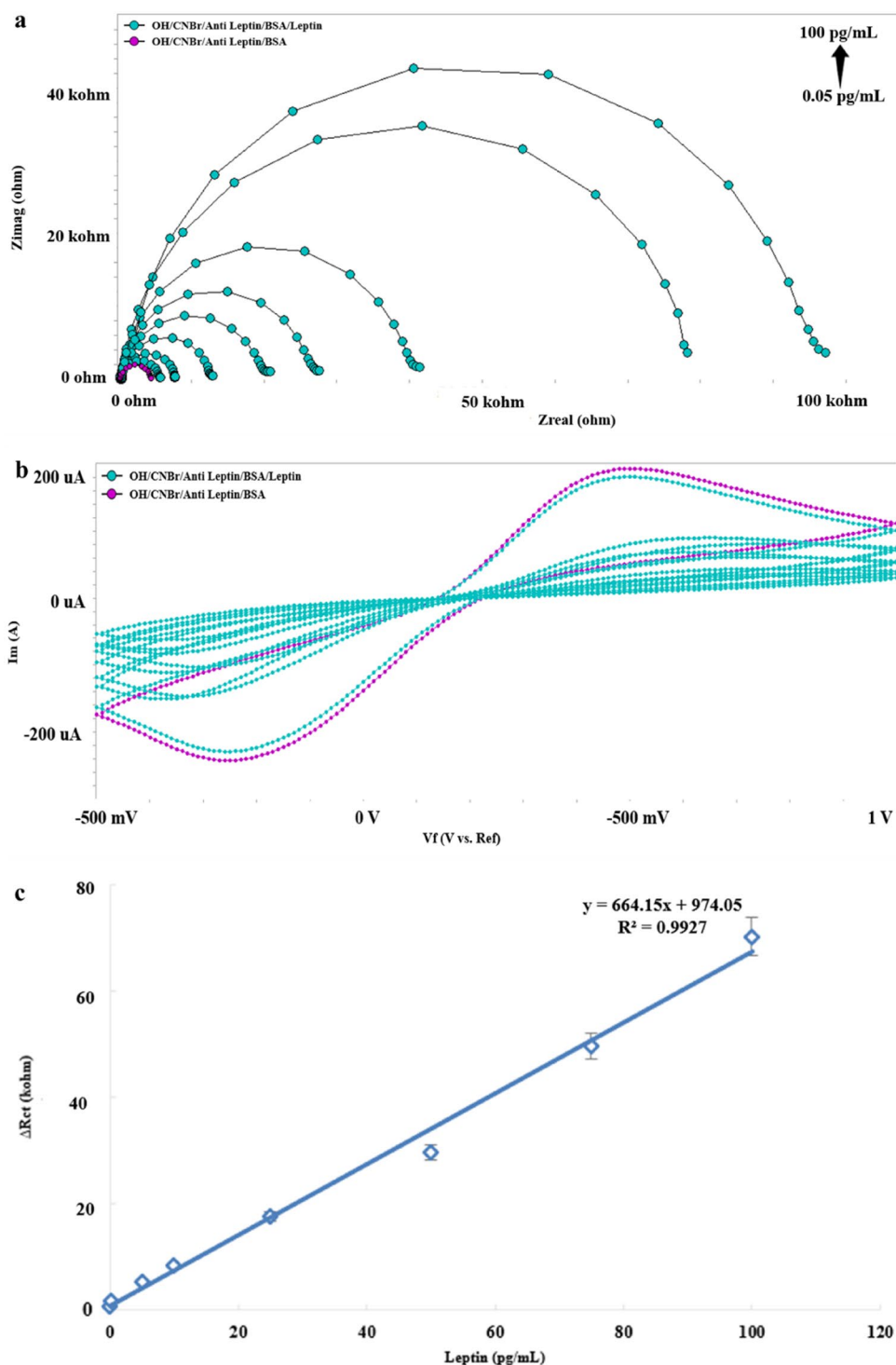
at increasing concentrations between 0.05 and 100 pg/mL after all immobilization steps and optimization processes were completed. Although the R_{ct} increased due to increasing antigen concentrations, the peak currents of CV decreased as expected. The calibration graph of the leptin biosensor designed in Fig. 2c is given. As can be seen, the biosensor has a broad linear detection range of 0.05–100 pg/mL. In addition, LOD and LOQ values were calculated as 0.0086 pg/mL and 0.0287 pg/mL, respectively. Although calculating the LOD and LOQ values, " $k \cdot S$ (standard deviation)/ m (slope of the curve)" equation was used. The value of k was accepted as 3 for LOD and 10 for LOQ (Demirbakan and Sezgentürk 2019).

Linear graphs are showing in S3a the reproducibility of the designed leptin biosensor system. For reproducibility, leptin biosensors were prepared by different persons at different times under the same conditions, and impedance measurements were carried out within the linear detection range (0.05–100 pg/mL). This process was repeated ten times. The relative standard deviation of the overlapping line graphs was calculated as 3.42%. The reproducibility study conducted independently proves the stability of the designed leptin biosensor.

The shelf life of the leptin immunosensor was determined in S3b. A robust biosensor should have a long shelf life. As a result, five weeks of storage measurements were conducted. At the same time, electrodes made under the same conditions were kept in the dark environment at +4° C. Each week, one of the electrodes was treated with 50 pg/mL leptin antigen for EIS measurement. No significant loss of activity was detected during the storage period for leptin measurement. One may say that this novel immobilization process results in forming a stable biosensor surface. Although the proposed biosensor is disposable, it has been evaluated for regeneration and shown to retain its high activity, as shown in S3 c. To regenerate the ITO-PET electrode, it was incubated for 45 min with leptin antigen (50 pg/mL) and then treated for five minutes with 0.1 percent HCl solution to disrupt the antibody–antigen interaction. Following each acid treatment, the surface was re-incubated with leptin antigen (50 pg/mL), and the impedance was measured. This regeneration procedure may be repeated up to 12 times. Finally, the acid destroyed the electrode surface, resulting in a significant drop in leptin binding ability. Nonetheless, the disposable biosensor's effectiveness and minimal error margins might be viewed as a cost-cutting measure.

When S3d is examined, it is seen that in the Kramers Kronig transformation, experimental data coincides with the virtual calculation of the components. The transformation was used to determine whether the impedance spectrum of the developed biosensor system was affected by external factors and deviations. (Özcan et al. 2014).

Fig. 2 Determination of leptin in increasing concentrations **a** EIS spectra **b** CV voltammograms **c** Calibration graph of leptin immunosensor

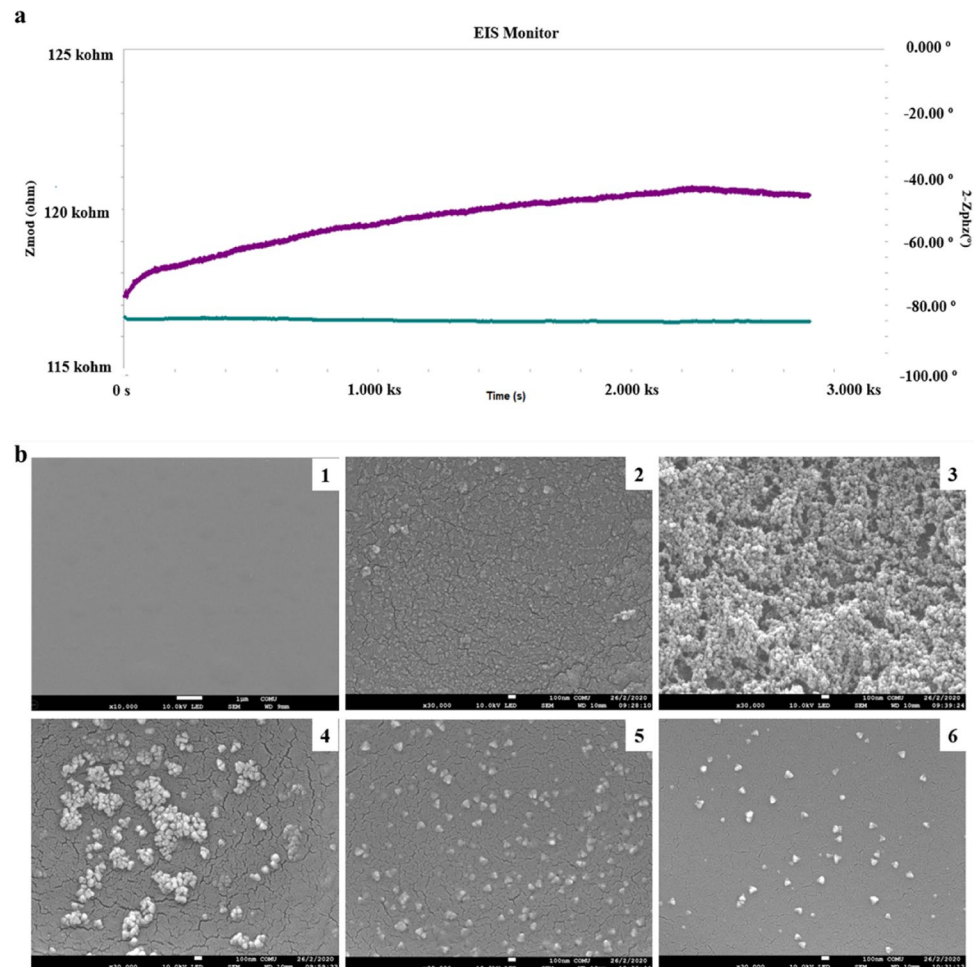


Repeatability is a critical parameter that indicates the biosensor's stability. Under the same conditions, twenty separate electrodes were created. The electrodes were incubated with leptin antigen at a 50 pg/mL concentration, which was chosen to be within the linear detection range for the measurements. The mean value, standard deviation, and coefficient of variation values were determined using the calibration

equation. The values are 48.89 pg/mL, ± 1.907 pg/mL and 3.9%, respectively. As a result, it can be inferred that the developed biosensor is highly repeatable.

The SFI methodology illustrated in Fig. 3a permits real-time monitoring of changes in the electrode surface caused by antigen–antibody interaction that are not observable during incubation. The picture shows that the surface

Fig. 3 Characterization studies for leptin immunosensor **a** Single-frequency impedance technique **b** Imaging of surface morphology of immobilization steps with SEM (1) Bare ITO-PET surface (2) Hydroxylated ITO-PET surface (3) CNBr treatment (4) Immobilization of anti-leptin (5) BSA treatment (6) Immobilization of leptin antigen



difference was measured concurrently by adding antigen to the Tris–HCl solution at a set frequency value determined from the Bode graph. Additionally, this approach provides an estimate of the antigen incubation period. SFI measurements were made during the optimization study-determined incubation time. 45 min of antigen incubation is adequate and essential for immobilization.

SEM was utilized to visualize the morphological changes on the electrode surface generated by each immobilization stage. The acquired pictures are shown in Fig. 3b. The surface of the ITO-PET electrode after cleaning is seen in Fig. 3b 1. The surface cleaned with an appropriate cleaning process is observed to be free of pollution. The rough surface in Fig. 3b 2 is caused by the hydroxyl groups immobilized on the surface during the hydroxylation process. Simultaneously, the surface is homogenous. Figure 3b 3 illustrates the immobilization of CNBr on the ITO-PET surface, which was attempted for the first time in the literature. The formation of a dense layer on the electrode surface is observed due to the efficient binding of CNBr to the hydroxyl groups. On this basis, the newly devised approach may be concluded to achieve the desired

immobilization. Figure 3b 4 shows that a new layer of spherical formations was detected on the surface following CNBr immobilization. The surface treatment with BSA is shown in Fig. 3b 5. Once again, it reveals that globular protein structures coat the surface uniformly. As seen in Fig. 3b 6, the final stage, leptin antigen immobilization, forms a new layer and sphere-like protein structures.

Applicability of leptin biosensor to real serum samples

The proposed biosensor's reaction to real serum samples was evaluated following analytical investigations. The studies performed in this research were authorized by the ethics council of noninvasive clinical research of Tekirdağ Namık Kemal University (Ethic committee approval number 2013/86/07/05). When variables such as relative standard deviation and recovery are evaluated in Table 1, they are computed. According to the obtained data, the margin of error is small, and the developed biosensor has a great potential for Leptin detection in the clinical setting.

Table 1 Applicability of leptin biosensor to real serum samples

Serum sample number	Leptin conc (pg/mL)	Standard added conc (pg/mL)	Measured conc (pg/mL, $n = 3$)	RSD% ($n = 3$)	Recovery (%)
1	21.00	10	31.18/30.59/29.85	1.48	101.48
		50	73.84/74.65/73.54	4.24	95.76
2	1.73	10	11.07/12.43/11.03	1.82	101.82
		50	46.64/49.52/51.22	5.02	105.02
3	11.29	10	23.02/21.18/22.11	3.84	96.16
		50	58.49/60.05/60.93	2.38	102.39
4	22.97	10	31.89/32.99/32.25	1.82	101.82
		50	72.14/67.54/70.92	3.80	103.8
5	18.89	10	28.41/29.21/28.92	0.16	100.16
		50	69.86/64.66/62.12	4.85	104.85

Conclusions

We require more cost-effective and uncomplicated immobilization procedures. Activation of cyanogen bromide has been restricted to earlier techniques. Its immobilization with antibodies is one of the ways that has not been the focus of thorough and well-studied research so far. With this study, using an innovative approach not included in the literature, an electrochemical immunosensor for leptin determination by CNBr activation of the ITO PET surface was designed by us for the first time. Efforts are currently being made to improve these techniques to promote enzyme binding, attain thermal stability, and circumvent the complicated and time-consuming procedures required to generate active resins (Sneha et al. 2019). Based on this, CNBr was used effectively and practically with this method based on antibody antigen-specific interaction, without any crosslinker or marker. The possibility of efficiently immobilizing such molecules without significant loss in their biological activities has been an important development. The precision, stability, and repeatability of the leptin immunosensor have been successfully characterized. The designed biosensor has a wide linear detection range (0.05–100 pg/mL, 2000 times), low LOD (0.0086 pg/mL) and LOQ (0.0287 pg/mL). Although it is disposable, it has been concluded that the ITO working electrode maintains its activity even in repeated studies. Finally, based on the test results in real human serum samples, it can be concluded that the designed biosensor system has a very high potential for early detection of medical treatments. This novel immobilization method was developed to allow the design of an electrochemical immunosensor with many different biomarkers in the future. Thus, this method is extremely suitable for immobilizing enzymes, antibodies, peptide hormones, and similar biologically active molecules.

Current biosensor research focuses on developing low-processing target analyte detection devices. It must fulfill all criteria, including near-perfection in technique, affordability,

ease of use, practical transportation, and being selective and extremely responsive. Each approach has limitations that need to be addressed in this regard. Today's rapidly evolving technology has a significant impact on the field of biosensors. The future will be guided by adapting biosensor systems to applications such as smartphones, miniaturization, and commercialization-ready technologies.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13205-021-03096-w>.

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Declarations

Conflict of 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.

References

- Azarikia F, Wu BC, Abbasi S, McClements DJ (2015) Stabilization of biopolymer microgels formed by electrostatic complexation: influence of enzyme (laccase) cross-linking on pH, thermal, and mechanical stability. *Food Res Int* 78:18–26. <https://doi.org/10.1016/j.foodres.2015.11.013>
- Bahadir EB, Sezgintürk MK (2015) Electrochemical biosensors for hormone analyses. *Biosens Bioelectron* 68:62–71. <https://doi.org/10.1016/j.bios.2014.12.054>
- Benbaibache H, Bounihi A, Koceir EA (2020) Leptin level as a biomarker of uncontrolled eating in obesity and overweight. *Ir J Med Sci*. <https://doi.org/10.1007/s11845-020-02316-1>
- Bosy-Westphal A, Müller MJ (2021) Diagnosis of obesity based on body composition-associated health risks—time for a change in paradigm. *Obes Rev* 22(S2):1–7. <https://doi.org/10.1111/obr.13190>
- Cai J, Gou X, Sun B, Li W, Li D, Liu J, Hu F, Li Y (2019) Porous graphene-black phosphorus nanocomposite modified electrode for

- detection of leptin. *Biosens Bioelectron* 137:88–95. <https://doi.org/10.1016/j.bios.2019.04.045>
- Campuzano S, Pedrero M, Yáñez-Sedeño P, Pingarrón JM (2021) New challenges in point of care electrochemical detection of clinical biomarkers. *Sens Actuators b: Chem.* <https://doi.org/10.1016/j.snb.2021.130349>
- Cavallo FR, Mirza KB, De Mateo S, Nikolic K, Rodriguez-Manzano J, Toumazou C (2021) Aptasensor for quantification of leptin through PCR amplification of short DNA-aptamers. *ACS Sensors* 6(3):709–715. <https://doi.org/10.1021/acssensors.0c02605>
- Chiou SH, Wu WT (2004) Immobilization of *Candida rugosa* lipase on chitosan with activation of the hydroxyl groups. *Biomaterials* 25(2):197–204. [https://doi.org/10.1016/S0142-9612\(03\)00482-4](https://doi.org/10.1016/S0142-9612(03)00482-4)
- Demirbakan B, Sezgentürk MK (2019) A novel electrochemical immunosensor based on disposable ITO-PET electrodes for sensitive detection of PAK 2 antigen. *J Electroanal Chem* 848:113304. <https://doi.org/10.1016/j.jelechem.2019.113304>
- Dey S, Murmu N, Bose M, Ghosh S, Giri B (2021) Obesity and chronic leptin resistance foster insulin resistance: an analytical overview. *BLDE Univ J Health Sci* 6(1):7. https://doi.org/10.4103/bjhs.bjhs_29_20
- Eleftheriou F, Takeda S, Ebihara K, Magre J, Patano N, Kim CA, Ogawa Y, Liu X, Ware SM, Craigen WJ, Robert JJ, Vinson C, Nakao K, Capeau J, Karsenty G (2004) Serum leptin level is a regulator of bone mass. *Proc Natl Acad Sci USA* 101(9):3258–3263. <https://doi.org/10.1073/pnas.0308744101>
- García-García P, Guisan JM, Fernandez-Lorente G (2020) A mild intensity of the enzyme-support multi-point attachment promotes the optimal stabilization of mesophilic multimeric enzymes: amine oxidase from *Pisum sativum*. *J Biotechnol* 318:39–44. <https://doi.org/10.1016/j.jbiotec.2020.04.006>
- Geissler M, Brassard D, Clime L, Pilar AVC, Malic L, Daoud J, Barrère V, Luebbert C, Blais BW, Corneau N, Veres T (2020) Centrifugal microfluidic lab-on-a-chip system with automated sample lysis, DNA amplification and microarray hybridization for identification of enterohemorrhagic: *Escherichia coli* culture isolates. *Analyst* 145(21):6831–6845. <https://doi.org/10.1039/d0an01232g>
- Ghadge AA, Khaire AA (2019) Leptin as a predictive marker for metabolic syndrome. *Cytokine* 121:154735. <https://doi.org/10.1016/j.cyt.2019.154735>
- Guisan JM (2013) Immobilization of enzymes and cells IN series editor. *Immobil Enzym Cells* 1051:1–375. <https://doi.org/10.1007/978-1-62703-550-7>
- Gupta R, Raza N, Bhardwaj SK, Vikrant K, Kim KH, Bhardwaj N (2021) Advances in nanomaterial-based electrochemical biosensors for the detection of microbial toxins, pathogenic bacteria in food matrices. *J Hazard Mater* 401:123379. <https://doi.org/10.1016/j.jhazmat.2020.123379>
- Kumar V (2005) Cyanogen bromide (CNBr). *Synlett* 10:1638–1639. <https://doi.org/10.1055/s-2005-869872>
- Ma D, Feitosa MF, Wilk JB, Laramie JM, Yu K, Leiendecker-Foster C, Myers RH, Province MA, Borecki IB (2009) Leptin is associated with blood pressure and hypertension in women from the National heart, lung, and blood institute family heart study. *Hypertension* 53(3):473–479. <https://doi.org/10.1161/HYPERTENSIONAHA.108.118133>
- Mihailescu CM, Stan D, Savin M, Moldovan CA, Dinulescu S, Radulescu CH, Firtat B, Muscalu G, Brasoveanu C, Ion M, Dragomir D, Stan D, Ion AC (2020) Platform with biomimetic electrochemical sensors for adiponectin and leptin detection in human serum. *Talanta* 210:120643. <https://doi.org/10.1016/j.talanta.2019.120643>
- Moradi N, Muhammadnejad S, Delavari H, Pournouri N, Oghabian MA, Ghafouri H (2021) Bio-conjugation of anti-human CD3 monoclonal antibodies to magnetic nanoparticles by using cyanogen bromide: a potential for cell sorting and noninvasive diagnosis. *Int J Biol Macromol* 192:72–81. <https://doi.org/10.1016/j.ijbiomac.2021.09.129>
- Özcan B, Demirbakan B, Yeşiller G, Sezgentürk MK (2014) Introducing a new method for evaluation of the interaction between an antigen and an antibody: single frequency impedance analysis for biosensing systems. *Talanta* 125:7–13. <https://doi.org/10.1016/j.talanta.2014.02.067>
- Peng B, Zhao F, Ping J, Ying Y (2020) Recent advances in nanomaterial-enabled wearable sensors: material synthesis, sensor design, and personal health monitoring. *Small* 16(44):1–24. <https://doi.org/10.1002/sml.202002681>
- Perumal K, Huin WK, Yap NY, Ong TA, Gobe GC, Rajandram R (2019) Role of leptin as a biomarker for early detection of renal cell carcinoma? No evidence from a systematic review and meta-analysis. *Med Hypotheses* 129:109239. <https://doi.org/10.1016/j.mehy.2019.109239>
- Randviir EP, Banks CE (2013) Electrochemical impedance spectroscopy: an overview of bioanalytical applications. *Anal Methods* 5(5):1098–1115. <https://doi.org/10.1039/C3AY26476A>
- Salek-Maghsoudi A, Vakhshiteh F, Torabi R, Hassani S, Ganjali MR, Norouzi P, Hosseini M, Abdollah M (2018) Recent advances in biosensor technology in assessment of early diabetes biomarkers. *Biosens Bioelectron* 99:122–135. <https://doi.org/10.1016/j.bios.2017.07.047>
- Sneha HP, Beulah KC, Murthy PS (2019) Enzyme immobilization methods and applications in the food industry. *Enzymes in food biotechnology*. Elsevier Inc, pp 645–658
- Sung R, Heo YS (2020) Sandwich ELISA-based electrochemical biosensor for leptin in control and diet-induced obesity mouse model. *Biosensors* 11(1):1–9. <https://doi.org/10.3390/bios11010007>
- Törer H, Aydın EB, Sezgentürk MK (2018) A label-free electrochemical biosensor for direct detection of RACK 1 by using disposable, low-cost and reproducible ITO based electrode. *Anal Chim Acta* 1024:65–72. <https://doi.org/10.1016/j.aca.2018.04.031>
- Wang W, Wang X, Cheng N, Luo Y, Lin Y, Xu W, Du D (2020) Recent advances in nanomaterials-based electrochemical (bio)sensors for pesticides detection. *TrAC-Trends Anal Chem* 132:116041. <https://doi.org/10.1016/j.trac.2020.116041>
- Yılmaz N, Aydın EB, Sezgentürk MK (2019) An epoxysilane modified indium tin oxide electrode for the determination of PAK 2: application in human serum samples. *Anal Chim Acta* 1062:68–77. <https://doi.org/10.1016/j.aca.2019.02.020>
- Zhang Q, Qing Y, Huang X, Li C, Xue J (2018) Synthesis of single-walled carbon nanotubes–chitosan nanocomposites for the development of an electrochemical biosensor for serum leptin detection. *Mater Lett* 211:348–351. <https://doi.org/10.1016/j.matlet.2017.10.036>