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Research Article

Design of a Novel Electrochemical Immunosensor for *Toxoplasma gondii* Detection Based on Gold Nanoparticle/Chitosan Decorated Screen Printed Electrode

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Abstract

Toxoplasma gondii is an intracellular parasite that primarily utilizes cats as its definitive host. This parasite, which is spread through cat feces, can be transmitted to other animals and, from there, to humans through various routes. In healthy individuals, this parasite may not cause severe illness, but it can be fatal, especially in individuals with weakened immune systems due to factors like chemotherapy or organ transplantation, as well as in fetuses developing in the womb. Given its relatively common occurrence worldwide, controlling the spread of this parasite is imperative. In this study, an antibody-based electrochemical biosensor was developed to directly detect *Toxoplasma gondii*, offering potential applications in disease surveillance and management. The biosensor was designed using screen-printed electrodes, and the electrode surface was modified with chitosan and gold nanoparticles. Anti-*Toxoplasma gondii* antibodies were immobilized onto the modified electrode surface using glutaraldehyde as a cross-linking agent. Changes in surface properties were investigated using various techniques, including differential pulse voltammetry, cyclic voltammetry, and electrochemical impedance spectroscopy. The surface morphology of the developed immunosensor was visualized using scanning electron microscopy. Subsequently, the biosensor's linear working range and detection limit were determined, followed by its application in the detection of *Toxoplasma gondii* in synthetic serum samples. This innovative approach holds promise for the development of sensitive and specific diagnostic tools for the detection of *Toxoplasma gondii* infection, which is important for effective disease prevention.
Keywords: *Toxoplasma gondii*, pathogen, biosensor, electrochemistry, immunosensor

Toksoplazma gondii Tespiti İçin Altın Nanoparçacık/Kitosan ile Dekore Edilmiş Serigrafi Baskı Elektrota Dayalı Yeni Bir Elektrokimyasal İmmünsensör Tasarımı

Öz

Toksoplazma gondii hücre içi bir parazittir ve ana konak olarak kedileri kullanmaktadır. Kedi dışkısı ile yayılan bu parazit diğer canlılara ve oradan insanlara birçok farklı yolla bulaşabilmektedir. Bu parazit, sağlıklı bireylerde ciddi hastalıklara yol açmayabilmektedir ancak özellikle kemoterapi veya organ nakli gibi faktörler nedeniyle bağışıklık sistemi zayıflamış bireylerde ve anne karnında gelişen fetüslerde ölümcül olabilmektedir. Bu parazit enfeksiyonunun tüm dünyada oldukça sık görülmesi sebebiyle kontrol altında tutulması gerekmektedir. Bu amaçla gerçekleştirilen çalışmada *Toksoplazma gondii*'yi direkt tespit edebilen antikor temelli elektrokimyasal bir biyosensör geliştirilmiştir. Geliştirilen sensörde serigrafi baskılı elektrotlar kullanılmıştır. Elektrot yüzeyi kitosan ve altın nanoparçacıklarla modifiye edilmiştir. Modifiye edilen elektrot yüzeyine anti-*Toksoplazma gondii* antikorları glutaraldehit ile immobilize edilmiştir. Yüzey özelliklerindeki değişimler diferansiyel puls voltametrisi, döngüsel voltametri, elektrokimyasal impedans spektroskopisi gibi tekniklerle incelenmiştir. Geliştirilen immünosensörün yüzey morfolojisi taramalı elektron mikroskopu ile görüntülenmiştir. Doğrusal çalışma aralığı ve tayin limiti belirlenen biyosensör daha sonra sentetik serum içerisinde *Toksoplazma gondii* tayininde kullanılmıştır. Bu yenilikçi yaklaşım, hastalığın etkili bir şekilde önlenmesi için önemli olan *Toksoplazma gondii* enfeksiyonunun tespitine yönelik hassas ve spesifik teşhis araçlarının geliştirilmesi konusunda umut vaat etmektedir.
Anahtar Kelimeler: *Toksoplazma gondii*, patojen, biyosensör, elektrokimya, immünosensör

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1. Introduction

Toxoplasma gondii (TOX) is an intracellular protozoan parasite responsible for causing a disease known as toxoplasmosis[1, 2]. This pathogen primarily utilizes members of the Felidae family, particularly domestic cats, as its definitive hosts, while it employs warm-blooded animals as intermediate hosts[3, 4]. TOX is found in the oocyst form in cat feces, which can be disseminated into the environment extremely fast. In humans, TOX infection is typically associated with the consumption of unwashed vegetables, undercooked meat, or contact with animal feces[5]. Recent studies also have presented potential evidence of sexual transmission of the disease[6, 7].

Toxoplasmosis in humans often manifests as an asymptomatic infection; however, depending on the individual's immune status, it can lead to serious consequences[1]. Particularly during the first trimester of pregnancy, transmission from mother to fetus can result in symptoms that may lead to fetal death[8–10]. In individuals with weakened immune systems, such as those with HIV, undergoing chemotherapy, or having undergone organ transplantation, TOX infection can cause neurological disorders, inflammation in various organs, and even death[11, 12]. Given that a large majority of humans and cats worldwide are infected, direct monitoring of this pathogen is of paramount importance[3, 11].

In Türkiye, TOX tests are generally conducted by examining patients' anti-Toxoplasma IgM and IgG values from their blood samples needed, to monitor acute infections or past infection history of the patients[13, 14]. However, direct determination of TOX is necessary for preventive measures before infection occurs[15].

In this study, the direct detection of TOX was achieved using electrochemical methods. The biosensor involved the modification of screen-printed electrodes (SPE) with chitosan and gold nanoparticles. Gold nanoparticles (AuNP) were employed to enhance the sensitivity of the sensor due to their biocompatibility and ability to improve charge transfer. Chitosan (CHT) is a polymer with free amine groups, commonly used in biosensors, which provide binding sites for biomolecules[16]. Anti-*Toxoplasma gondii* antibodies (Ab_{TOX}) were immobilized onto the electrode surface using glutaraldehyde through these amine groups. Immobilized Ab_{TOX} captured *Toxoplasma gondii* proteins on the surface, which were then determined using differential pulse voltammetry. Surface modifications were characterized using cyclic voltammetry, electrochemical impedance spectroscopy, and UV-visible spectrophotometry. Scanning electron microscopy was employed for morphological characterization.

2. Material and Methods

2.1 Instrumentation and Chemicals

All electrochemical measurements such as cyclic voltammetry (CV), differential pulse voltammetry (DPV), and electrochemical impedance spectroscopy (EIS) were carried out with PalmSens 4 potentiostat, Electrochemical impedance spectroscope driven by PC Trace 5.9

software (Houten, Netherland). 4 mm carbon screen-printed carbon electrodes were obtained from Life Sense Teknoloji (Samsun, Türkiye). Surface morphology studies of modified electrodes were carried out with Hitachi SU-1510 scanning electron microscope (SEM) (Japan). The UV-visible spectrophotometer was used for the examination of CHT-AuNP interaction. Anti-toxoplasma antibody and toxoplasma protein were obtained from Abcam (Cambridge, UK). All other analytical grade chemicals were obtained from Sigma Aldrich (Missouri, ABD).

2.2 Construction of Biosensor

AuNP synthesis

100 mL of 100 µM $HAuCl_4$ was poured into a round bottom flask and heated until boiling temperature. 3 mL of 1% sodium citrate was added to the hot $HAuCl_4$ solution with rapid magnetic stirring. The solution color was turned from pale yellow to bright red, indicating gold nanoparticle formation[17]. The nanoparticle solution was heated and mixed for 15 minutes more to complete the reaction.

Electrode Modification

Screen printed electrodes (SPE) with 4 mm carbon working electrodes were precleaned with 10 cyclic voltammetry measurements between -2 V and +2 V in 0.05 M HCl solution before modifications. 7.5 µL of CHT and AuNP mixture was drop-casted on a carbon working electrode and dried on a hot plate at 50 °C. The surface was washed three times with 1 mL of pH:7.4, 50 mM phosphate buffered saline solution (PBS) to remove unbounded chemicals and labeled as SPE/CHT-AuNP. Amino groups of SPE/CHT-AuNP surface were activated with 7.5 µL of 1 % glutaraldehyde (GA) crosslinker for 1 hour at 4 °C. The surface was washed three times with 1000 µL PBS to remove unreacted GA and dried with nitrogen gas. Afterward, 7.5 µL of Ab_{TOX} was added to the surface and let the reaction overnight at 4 °C[18]. GA crosslinker created the covalent bond between the primary amine groups of antibody and chitosan amine groups. Unbounded Ab_{TOX} was removed from the surface by washing three times with PBS-Tween 20 solution and the surface labeled as SPE/CHT-AuNP/Ab_{TOX}. 10 µL of 1 % Bovine Serum Albumin (BSA) solution was added at SPE/CHT-AuNP/Ab_{TOX} surface and washed after 30 minutes with PBS-Tween 20. BSA was used as a surface-blocking agent to prevent nonspecific interactions. The calibration curve of the biosensor was obtained after optimization studies. Biosensor modification steps were summarized in Figure 1.

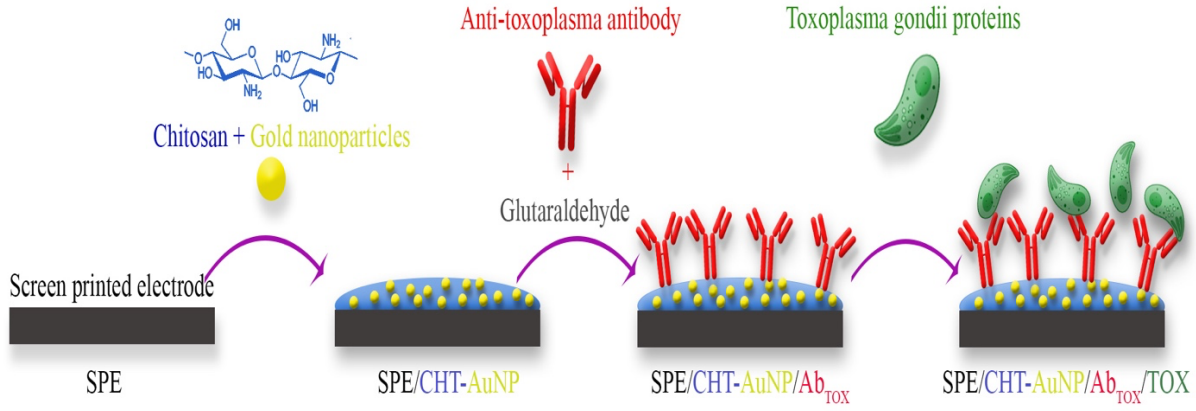

Figure 1. Biosensor construction.

Electrochemical Measurements

DPV which is a very sensitive electrochemical method was used for the detection of TOX. 1 mM $K_3[Fe(CN)_6]^{-3,-4}$ in 0.1 M KCl solution (HCF) was used as a redox mediator for all TOX detection experiments, and surface characterization studies. DPV peaks of the HCF solution were measured between -0.35 V and 0.65 V potentials before and after interacting the SPE/CHT-AuNP/Ab_{TOX} surface with TOX. The difference between the peak potentials of SPE/CHT-AuNP/Ab_{TOX} and SPE/CHT-AuNP/Ab_{TOX}/TOX was taken as biosensor response.

Optimizations

The optimization of Ab_{TOX} concentration was performed on the SPE/CHT surface. Antibody concentrations ranging from 10 µg/mL to 100 µg/mL were immobilized on electrodes, and subsequent biosensor responses were assessed and compared. The Ab_{TOX} concentration yielding the highest biosensor responses was considered the optimal concentration. Following this, solutions of AuNPs ranging from 5 µL to 30 µL were combined with CHT and applied to SPEs' surfaces. Variations in SPE/CHT-AuNP/Ab_{TOX} responses were then analyzed in relation to the incremental addition of AuNPs.

Characterization Studies

The techniques of CV and EIS were employed for electrochemical characterization. Experiments were carried out by adding an 80 µL of 5 mM HCF solution onto the biosensor surface. A scanning electron microscope (SEM) was utilized to observe surface morphology and capture images at various magnifications throughout all stages of surface modification. Characterization of the CHT-AuNP nano-composite was carried out using UV-VIS spectrophotometry.

Real sample experiments

In real-sample experiments, synthetic serum containing *Toxoplasma gondii* proteins was chosen over *Toxoplasma gondii* because of high pathogenicity. The composition of the synthetic serum was provided in Table 1[19]. The prepared synthetic serum was spiked with 25 ng/mL TOX and examined on the SPE/CHT-AuNP/Ab_{TOX}.

Table 1. Synthetic serum composition

Compound	Concentration (mM)
$MgCl_2$	1.6
NH_2CONH_2 (urea)	2.5
KCl	4.5
D(+)-Glucose	4.7
$CaCl_2$	5
NaCl	145
Human Serum Albumin	0.1 % (w/v)

3. Results and Discussion

While developing the biosensor surface, the initial objective was to ascertain the quantity of antibodies. During this stage, experiments were carried out on a CHT-coated surface with antibody concentrations ranging from 10 µg/mL to 100 µg/mL. While the signals at 10 µg/mL and 25 µg/mL Ab_{TOX} concentrations were notably low, the optimal biosensor response was obtained at 50 µg/mL Ab_{TOX} (Figure 2A). The signal decrease observed at 100 µg/mL Ab_{TOX} was attributed to a reduction in the interaction between the analyte and the surface due to the excessive accumulation of antibodies. 50 µg/mL Ab_{TOX} was used in the subsequent stages of the experiments.

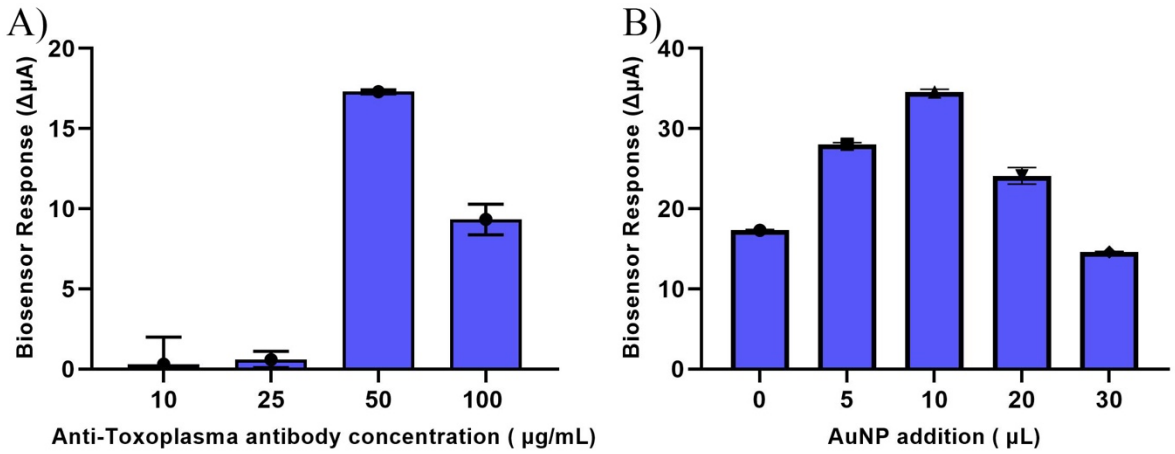

Figure 2. Biosensor response over different concentrations of Anti-toxoplasma antibody (A) and gold nanoparticle (B).

At the next optimization step, AuNPs were added to the CHT solution in volumes ranging from 5 µL to 30 µL to investigate their effects (Figure 2B). The highest biosensor response was obtained when 10 µL of AuNPs was added and was selected as the optimum value. It was observed that AuNPs enhanced the biosensor response due to their high conductivity properties[20–22]. The observed biosensor response decreased with an increase in the amount of AuNPs added could be attributed to the disruption of antibody orientations.

The electrochemical evaluation of electrode modifications has been demonstrated with CV and EIS techniques. Initially, the electrochemical behaviors of SPE, followed by SPE/CHT-AuNP, SPE/CHT-AuNP/Ab_{TOX}, and SPE/CHT-AuNP/Ab_{TOX}/TOX sequential stages, were assessed based on the current responses observed in CV measurements. The variations in anodic and cathodic current peaks were monitored across each modification step. The cathodic peak current magnitude for the unmodified SPE was recorded at -28.3 µA. In contrast, the SPE modified with CHT-AuNP exhibited a cathodic peak current of -61.9 µA. Further modification with the Ab_{TOX} on SPE/CHT-AuNP resulted in a cathodic peak current of -51.3 µA, while the subsequent introduction of the TOX on SPE/CHT-AuNP/Ab_{TOX} yielded a cathodic peak current of -39.3 µA. Conversely, the observed anodic peak current values were 21.0 µA for the unmodified SPE, 69.6 µA for SPE/CHT-AuNP, 50.1 µA for SPE/CHT-AuNP/Ab_{TOX}, and 33.1 µA for SPE/CHT-AuNP/Ab_{TOX}/TOX. The measured peak-to-peak separations were 0.104 V for bare SPE, 0.139 V for SPE/CHT-AuNP, 0.143 V for SPE/CHT-AuNP/Ab_{TOX}, and 0.139 V for SPE/CHT-AuNP/Ab_{TOX}/TOX.

As observed in Fig. 3A, in accordance with modified surfaces, it has been determined that the bare SPE surface generates significantly lower current peaks. After the surface coating with CHT-AuNP, a substantial enhancement in both anodic and cathodic peaks was observed. The presence of amine groups on the CHT surface, along with the conductivity-imparting AuNPs, was found to facilitate this increase in current. This behavior is analogous to prior studies where positively charged amine groups on polyethyleneimine-coated surfaces had a similar effect[23].

Furthermore, it was observed that the modification of the surface with antibodies and the binding of TOX proteins to the surface impeded electron transfer, thereby reducing both anodic and cathodic peak currents.

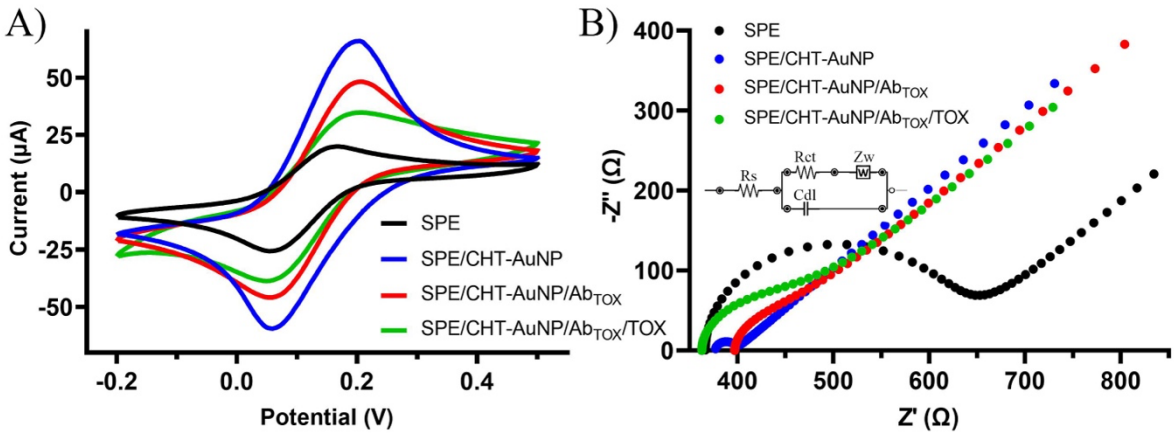

Figure 3. CV (A) and EIS (B) diagrams of SPE/CHT-AuNP/Ab$_{TOX}$/TOX sensor surface modification steps.

The electrochemical characterization of the SPE/CHT-AuNP/Ab$_{TOX}$ sensor was also examined using the EIS technique. Raw EIS data were fitted using the Randle equivalent circuit commonly employed in previous studies[16, 23, 24]. In Figure 3B, Nyquist plots were shown for each modification step. The Randle equivalent circuit was incorporated as an inset graph, and it displayed parameters including the solution resistance (Rs), Warburg impedance (Zw) resulting from the diffusion of the $[Fe(CN)_6]^{(3-/4-)}$ redox probe, the double-layer capacitance (Cdl), and the charge transfer resistance (Rct). The Rct value of bare SPE was 252.2 Ω. The Rct was significantly decreased to 20.5 Ω with SPE/CHT-AuNP surface. Increasing Rct were obtained respectively 55.1 Ω for Ab$_{TOX}$ immobilized and 91.0 Ω for TOX captured sensor surface.

SEM technique was used for topographical characterization of modification steps of the SPE/CHT-AuNP/Ab$_{TOX}$ sensor. Observations revealed that a single CHT layer created a smooth appearance on the surface. Upon the addition of AuNPs, they were visualized as bright spherical particles on the surface. Immobilization of Ab$_{TOX}$ led to a blurring effect in the images of AuNPs observed on the surface. The immobilization of TOX proteins by antibodies resulted in the formation of a significantly rough protein layer on the biosensor surface (Figure 4).

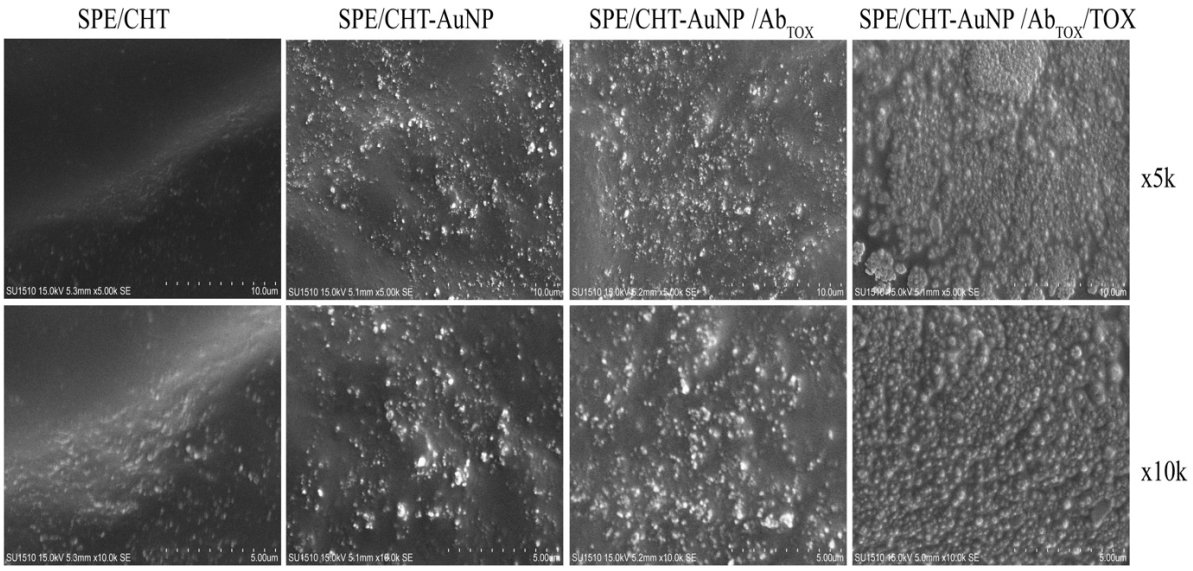

Figure 4. SEM images of SPE/CHT, SPE/CHT-AuNP, SPE/CHT-AuNP/Ab$_{TOX}$, and SPE/CHT-AuNP/Ab$_{TOX}$/TOX with 5k and 10k magnifications.

Additionally, the interaction of CHT with AuNP was observed with a UV-VIS spectrophotometer. In the UV-VIS spectra of AuNPs, a classic AuNP absorbance peak was observed at 520 nm[17]. On the other hand, the absorbance peak of CHT appeared as a broader peak at 300 nm[25]. In the CHT-AuNP mixture, the CHT peaks shifted to 280 nm, which indicates the formation of CHT nanoemulsion with AuNPs[26]. The absence of any shift in the AuNP peaks confirmed that there was no aggregation occurring (Figure 5).

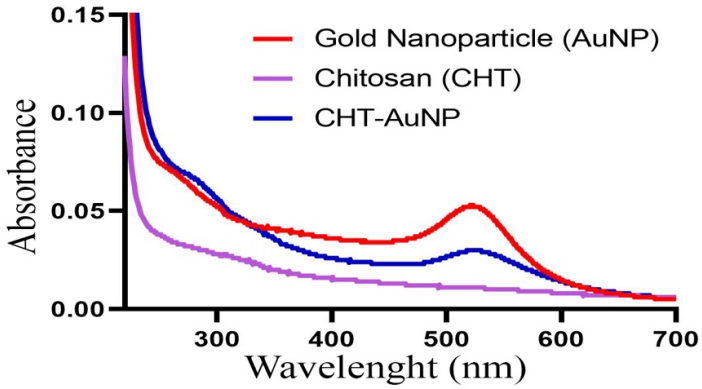

Figure 5. UV-Visible spectrums of AuNP, CHT, and CHT-AuNP.

To assess the analytical capabilities of the SPE/CHT-AuNP/Ab$_{TOX}$ electrode for TOX detection, the DPV technique was employed. Various concentrations of TOX were applied to the electrode, and the evaluation hinged on the current signals derived from the disparity between the peak responses of SPE/CHT-AuNP/Ab$_{TOX}$ in the presence and absence of the analyte (TOX ranging from 1.0 to 100 ng/mL).

Following the introduction of varying TOX concentrations to the SPE/CHT-AuNP/Ab_{TOX} electrode, a decrement in DPV signals was observed, as depicted in Figure 6A. Moreover, a standard addition curve over the range of 1-250 ng/mL TOX was demonstrated in Figure 6B. A linear standard curve was established over the 1-100 ng/mL of TOX range, yielding the equation y = 0.246x+7.483 (R2 = 0.9998), as illustrated in Figure 6B inset. Given the extensive dynamic range and the heightened sensitivity of this current biosensor, its potential as a promising candidate for *Toxoplasma gondii* detection is evident.

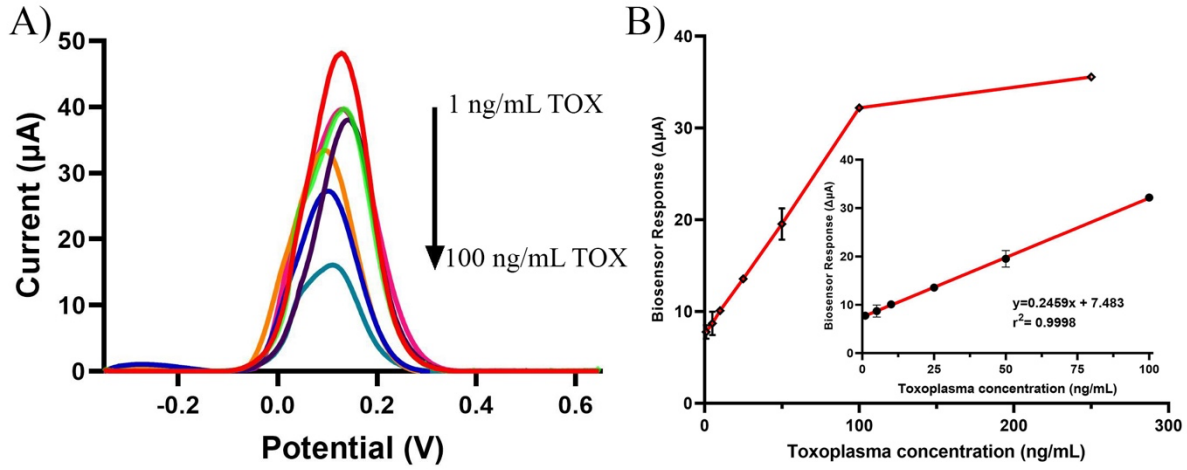

Figure 6. DPV signals of TOX ranging from 1.0 to 100 ng/mL (A), standard addition curve over the range of 1-250 ng/mL TOX (B), and linear standard curve of SPE/CHT-AuNP/Ab_{TOX} (B inset).

The repeatability and limit of detection (LOD) of the SPE/CHT-AuNP/Ab_{TOX} platform were also investigated. The repeatability was assessed through six consecutive measurements, yielding a ± 0.025 % standard deviation and a coefficient of variation (cv) of 1.2 %. Regarding TOX LOD, it was determined using the 3.3SD/m formula, resulting in a value of 0.34 ng/mL (n = 6), based on the standard deviation of the intercept and the slope of the calibration curve.

The concentrations of TOX-spiked synthetic samples at 25 ng/mL were quantified using our biosensor, as outlined in the experimental section. The 26.42 ± 3.6 ng/mL concentration was obtained. These findings demonstrate the remarkable sensitivity of our biosensor relative to the conventional methods.

Table 2. Comparison of analytical properties of designed SPE/CHT-AuNP/AbTOX with other *Toxoplasma gondii* and toxoplasmosis detection techniques.

Method	Analyte	Linear range	LOD	Ref.
PCR	*Toxoplasma gondii* genomic DNA	5 × 101-2.5 × 104 fg/µL	5 fg/µL	[27]
PCR	*Toxoplasma gondii* genomic DNA	100 ng μL^{-1} - 400 ng μL^{-1}	100 ng μL^{-1}	[28]
Electrochemistry	*Toxoplasma gondii* genomic DNA	0.5 µg/mL -25 µg/mL	1.78 µg/mL	[29]
Colorimetric immunoassay	*Toxoplasma* lysate antigen antibodies	Dot-blot assay	-	[30]
Electrochemistry	anti-*Toxoplasma gondii* specific antibodies	0 U mL^{-1}- 200 U mL^{-1}	0.012 U mL^{-1}	[31]
Electrochemistry	*Toxoplasma gondii* proteins	1.0 - 100 ng/mL	0.34 ng/mL	This work

Table 2 provides a summary of several studies conducted on *Toxoplasma gondii* and anti-*Toxoplasma gondii* antibodies. Some of these studies have achieved a low limit of detection and high sensitivity. Notably, most of these techniques could be considered expensive measurement systems, and some of these studies have pointed out that the presence of other parasites in the sample can lead to interference. The developed SPE/CHT-AuNP/AbTOX biosensor in our research offers an economical, practical, and field-applicable solution compared to other methods, primarily owing to the utilization of screen-printed electrodes.

4. Conclusion

In this study, a novel electrochemical immunosensor capable of direct *Toxoplasma gondii* detection was designed. The designed sensor utilized screen-printed electrodes, which are highly practical for on-site detection, and a chitosan-gold nanoparticle nanocomposite on the electrode surface to enhance sensitivity and accuracy. The SPE/CHT-AuNP/Ab_{TOX} biosensor exhibited excellent analytical performance in the determination of TOX within a linear range of 1-100 ng/mL. Toxoplasmosis tests are typically developed by detecting the presence of anti-Toxoplasma antibodies in patients. However, the developed SPE/CHT-AuNP/Ab_{TOX} immunosensor allows for the direct detection of *Toxoplasma gondii* itself. Detecting disease sources prior to individuals contracting toxoplasmosis can be possible with this approach. Specially results of this work hold promise for the potential direct determination of *Toxoplasma gondii* from cat feces in future studies. In the context of domestic cats, there exists a slightly lower incidence of toxoplasmosis in comparison to stray feline populations.

Hence, routine vaccination and parasite control of household cats are of paramount importance for human health. Indeed, within this context, the designed biosensor exhibits the potential to evolve into a commercial product, offering a point-of-care diagnostic solution for the monitoring of *Toxoplasma gondii* in cat feces.

Ethics in Publishing

There are no ethical issues regarding the publication of this study.

Author Contributions

Serdar ŞANLI: The author confirms sole responsibility for the following: study conceptualization, methodology, validation, formal analysis, investigation, resources, writing - original draft, writing - review & and editing, visualization supervision, project administration, and funding acquisition.

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