Received: 2 January 2020 | Revised: 15 January 2020 | Accepted: 23 January 2020

DOI: 10.1002/jmr.2839

RESEARCH ARTICLE

Journal of Molecular Recognition WILEY

Binding of *Leishmania spp* with gold nanoparticles supported polyethylene glycol and its application for the sensitive detection of infectious photogenes in human plasma samples: A novel biosensor

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Abstract

The management of pathogen detection using a rapid and cost-effective method presents a major challenge to the biological safety of the world. The field of pathogen detection is nascent and therefore, faces a dynamic set of challenges as the field evolves. Visceral leishmaniasis (VL), or kala-azar is the most severe form of leishmaniasis. Delay to the accurate diagnosis and treatment is likely to lead to fatality. The reliable, fast and sensitive detection is closely linked to safe and effective treatment of *Leishmania spp*. Despite several routine and old method for sensitive and specificity detection of *Leishmania spp*, there is highly demand for developing modern and powerfully system. In this study a novel ultra-sensitive DNA-based biosensor was prepared for detection of *Leishmania spp*. For the first time, the specific and thiolated sequences of the *Leishmania* spp genome (5′-SH-$[CH_2]_6$ ATCTCGTAAGCAGATCGCTGTGTCAC-3′) were recognized by electrochemical methods. Also, selectivity of the proposed bioassay was examined by three sequences that were mismatched in 1, 2, and 3 nucleotides. The linear range (10^{-6} to 10^{-21} M) and limit of detection (LLOQ = 1 ZM) obtained are remarkable in this study. Also, simple and cost-effective construction of genosensors was another advantage of the proposal DNA-based assay. The experimental results promise a fast and simple method in detection of kala-azar patients with huge potential of the nanocomposite-based probe for development of ideal biosensors.

KEYWORDS

advanced nanomaterial, bioanalysis, chemical binding, hybridization, *Leishmania spp*, nucleic acid

1 | INTRODUCTION

Leishmania is a protozoan parasite and is of veterinary and medical significance. Visceral leishmaniasis (VL) is the most important type which is typically known as kala-azar and is the most severe form of leishmaniasis.[1] In the most cases, *Leishmania* leaves the inoculation place and proliferates in organs such as the spleen, liver and bone marrow. So, in the host, immunosuppression status can lead to death with the absence of treatment.[2] Therefore, rapid and specific diagnosis of *Leishmania* has an undeniable role in the treatment and control of related disease. Molecular based methods as well as polymerase chain reaction (PCR), real-time PCR, and qPCR are the most important and frequently used in identification of *Leishmania*.[3] For example, Weirather and co-workers applied the qPCR technique for rapid and

sensitive detection of *Leishmania*. Findings revealed that a developed method was a sensitive assay and can be used to detect *Leishmania* species infections in both clinical or experimental specimens and authorized to discriminate between the different *Leishmania* species.[4] A real-time PCR technique using SYBR Green I and primers was applied for detection of *Leishmania* in mouse tissue.[5] Results demonstrated that the real-time PCR technique is well-matched with the clinical diagnosis and will be useful to scientists not only in monitoring of the efficacy of anti-*Leishmanial* drugs and vaccines but also helpful in the study of the life cycle properties of *Leishmania* spp. Five species-specific PCR technique was performed for sensitive identification of *Leishmania donovani* in from of kala-azar and post-kala-azar patients. This method provides a valuable tool for the detection and simultaneous typing of *Leishmania* while diagnosis was performed on clinical samples. Similar tools are necessary to complement diagnostic methods and are used in clinical and veterinary approaches for early detection and simultaneous typing.[6] Although molecular based methods are important and extensively used technique but these methods have some disadvantages as well as, need for expensive and advanced equipment, necessary for professional people and difficult interpretation. For these reasons, and overcome molecular based methods restrictions, advanced and novel method established broadly in recent years. Biosensor technology is one of the most important approaches in detection of a wide range of analytes as well as drugs, proteins, enzymes and nucleic acid in medical applications. Biosensors are analytical devices with high sensitivity, simple construction and cost-effective aspects used in the field of microbiology. In linked parasitology and particularly *Leishmania*, various types of biosensors have been established in last decades. An innovative biosensor based on the surface plasmon resonance (SPR) immunosensor was established to detect C1 protein with an unknown function in *Leishmania infantum*. The platform was based on layer-by-layer assembly and designed by cysteamine in combination with a poly (amidoamine) dendrimer (PAMAM(G4)) on a gold surface to immobilize the protein. Biosensors were developed with high sensitivity and selectivity and were applicable in the detection of *L infantum C1* protein,[7] For accurate discrimination between cutaneous leishmaniosis and Chagas disease, an appropriate advanced biosensor was developed. A nanostructured system was able to detect specific anti-*Leishmania* antibodies with acceptable sensitivity and selectivity.[8] A nano-structured genosensor based on nickel oxide was developed for the detection of kala-azar. In this work, 18S rRNA gene sequences from *L donovani* were used as a specific target.[9] Also, an impedimetric genosensor based on gold nanoparticles (AuNPs) was developed for detection of *L infantum*. Electrochemical impedance spectroscopy (EIS) was used to identify *L infantum* and was applicable in samples acquired from contaminated dogs.[10] A magnetic bead/gold nanoparticle-based genosensor was established for detection of *Leishmania* DNA. In this work, AuNPs were used to label primers and magnetic beads were applied for sensitive and selective electrochemical detection. The findings showed much more sensitivity than molecular-based methods such as real-time PCR with rapid, low-priced, and easy properties.[11] A thiolated carbon nanotube (ThNT) transducer was used for ultra-detection of *Leishmania* spp. This was a simple and cost-effective genosensor for detecting canine leishmaniosis.[12] A SPR-based biosensor on mono/polychromatic sources was proposed for highly specific and sensitive detection of *Leishmania* chagasi antigens. The rapid operation time with high sensitivity and good specificity was reported for this biosensor.[13] In another report, a gold nanoparticle-based biosensing strategy was developed for sensitive detection of *Leishmania* DNA genome with both visual and spectrophotometric techniques. A PCR-free system showed acceptable sensitivity and was usable for detection of *Leishmania* DNA in clinical samples.[14] Recently developed biosensors for detection of *Leishmania* spp are shown in in Table 1.

AuNPs have been extensively used in biomedical applications due to their properties such as plasmon band localization in the visible spectrum, ease of synthesis, and numerous functional abilities. The size and properties of nanoparticles make them excellent platforms for the detection and identification of pathogens in native biological samples. To date, AuNP-based sensors were developed to detect different targets.[15-19] Moreover, AuNPs have been used as a platform for biomolecule immobilization. Their interactions with light facilitate their involvement with analytical applications. The optical properties of nanoparticles change according to their size and shape leading to different absorption bands. Due to the unique properties of nanoparticles, AuNPs are used to increase the sensitivity of diagnosis. The fundamental idea in the present work is based on DNA hybridization and target sequence detection via the spectrophotometric method.

Following the development of biosensors, a modern, ultra-sensitive genosensor was developed to identify specific sequences of *Leishmania*. In this research work, the thiolated sequence of the *Leishmania* genome was immobilized on the surface of gold electrodes and

TABLE 1 Some developed biosensors for detection of *Leishmania* spp

Type of pathogen	Types/methods	Linear range	LOD	References
Infantum	Immunosensor/SPR	9.70 to 51.8 nmol L^{-1}	7.37 nmol L^{-1}	7
Amazonensis	Immunosensor	1×10^{-5} mg mL^{-1}	10-5 mg mL^{-1}	8
Donovani	Genosensor/electrochemical	2 pg mL^{-1} to 2 mg mL^{-1}	2 pg mL^{-1}	9
Infantum	Impedimetric/electrochemical	—	0.01 pg mL^{-1}	10
Leishmania spp	Impedimetric/genosensor	3×10^{-13} to 3×10^{-10} M	6.6 PM	12
Infantum	Immunosensor/SPR	10-50 mg mL^{-1}	—	15
Leishmania spp	Genosensor/electrochemical	0.04-0.086 ng L^{-1}	7.0 pg L^{-1}	14
Leishmania spp	Genosensor/electrochemical	1 μM to 1 ZM	1 ZM	This work

used for hybridization with cDNA. Also, AuNPs were used for signal amplification and increased sensitivity. In addition, graphene quantum dots were used for electrochemical enhancement, too. In this study a novel ultra-sensitive DNA-based biosensor was prepared for detection of *Leishmania* spp. For the first time, the specific and thiolated sequences of the *Leishmania* spp. genome were recognized by electrochemical methods. The selectivity of the proposed bioassay was also examined by three sequences that mismatched in 1, 2, and 3 nucleotides. A simple and cost-effective construction of genosensors were another advantage of the proposal DNA-based assay.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Oligonucleotide sequences as well as designed probe for *Leishmania*: 5′-SH-$(CH_2)_6$ ATCTCGTAAGCAGATCGCTGTGTCAC-3′; complementary target sequence 5′-GTGACACAGCGATCTGCTTACGAGAT-3′; single nucleotide mismatch target sequence; 5′-CTGACACAGCGAT CTGCTTACGAGAT-3′; two nucleotide mismatch target sequences 5′-CCGACACAGCGATCTGCTTACGAGAT-3′; and three mismatch target sequences 5′-CCGACACAGCGATCTGCTTACGAGAT 3′ were provided by Takapouzist Co. (Iran). All of the oligonucleotide stock solutions were diluted with a 0.1 M Tris-HCl buffer, pH 7.4 solution. Potassium ferrocyanide $K_4Fe(CN)_6$, potassium ferricyanide $K_3Fe(CN)_6$, dttiothereitol (DTT), sodium acetate, toluidine blue (TB), hydrogen tetrachloroaurate(III)hydrate ($HAuCl_4{\cdot}3H_2O$), mercaptoethanole (MCE), were acquired from Sigma-Aldrich (Ontario, Canada). A ferricyanide/ferrocyanide solution comprising 0.5 M KCl and 0.5 M of $K_4Fe(CN)_6/K_3Fe(CN)_6$ (1:1) was used as a supporting electrolyte for the electrochemical measurement. Piranha solution was prepared by mixing hydrogen peroxide and concentrated sulfuric acid in a 1:2 (v/v) ratio. It was almost immediately to maintain its reactivity. A stock solution of 20 μM TB was prepared in Tris-HCl and employed as a redox indicator for revealing DNA hybridization. All of the above solutions were kept at −4°C before use. DTT solution comprising 10 mM sodium acetate and 500 mM of DTT, pH 5.2 was prepared and also kept at −4°C.

2.2 | Instrument

A PalmSens 4c system and three-electrode (from Metrohm) were employed for electrochemical measurements. Platinum wire and Ag/AgCl-Sat'd KCl were employed as counter and reference electrodes, respectively. The glass carbon electrode (GCE) 2 mm in diameter (from Azar Electrode Co., Urumia, Iran) was also employed as a working electrode. The electrochemical system was driven with PSTrace5 software. FE-SEM (emission scanning electron microscope; Hitachi-SU8020, Czech) with an operating voltage of 3 kV was used to evaluate the particles' morphology and modified electrode surface. For square wave voltammetry (SWV) measurements; E_{pulse} = 0.005 V, pulse amplitude = 0.001 V, and frequency = 10.0 Hz, and for differential pulse voltammetry measurements; E_{pulse} = 0.005 V, t_{pulse} = 0.2 second, scan rate = 1 V s^{-1}, and step potential = 0.1 V were applied.

3 | RESULTS AND DISCUSSION

3.1 | Electrode modification

Modification of the Au electrode surface plays a very important role in biosensor preparation so any weaknesses at this stage will have a direct impact on the quality of the system. Polishing of the gold electrode surface is very important for the system's performance. For this purpose, the surface of the Au electrode was polished on a piece soaked and impregnated with velvet alumina. In the next step, the gold electrode was placed in a piranha solution containing 0.5 mM H_2SO_4 and H_2O_2 for 5 minutes. After being washed with distilled water and placed in a solution of water:acetone (1:1), the gold electrode was ready for modification. The nanomaterial used in the present study included: $HAuCl_4$ (320 mM), H_2SO_4 (0.5 mM) and for increased stability of AuNPs, polyethylene glycol (1 M) was applied.[20,21] Then, prepared nanomaterial was used for gold electrode modification with a single step chronoamperometry technique in a solution of $K_4Fe(CN)_6/K_3Fe(CN)_6$ (Figure 1).

3.2 | Characterization of the materials and modified substrate

FE-SEM was used to determine the size and morphological evaluation of the biosensor preparation. The size and shape of AuNPs-Au electrode (Figure 2), biosensor before (Figure S1, see Supporting Information) and after hybridization with cDNA (Figure S2) were evaluated by FE-SEM technique. The results show the successful electrosynthesis of AuNPs on the surface of gold electrode in the nano-sized range and fixed form. The finding also shows the proper immobilization of pDNA and its hybridization with cDNA.

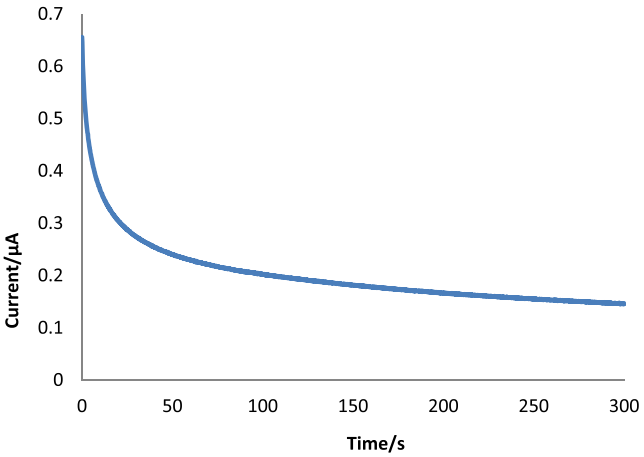


FIGURE 1 Single step chronoamperograms for the electrochemical deposition of nanomaterial on the surface of gold electrode. E = 0 V and t = 5 minutes

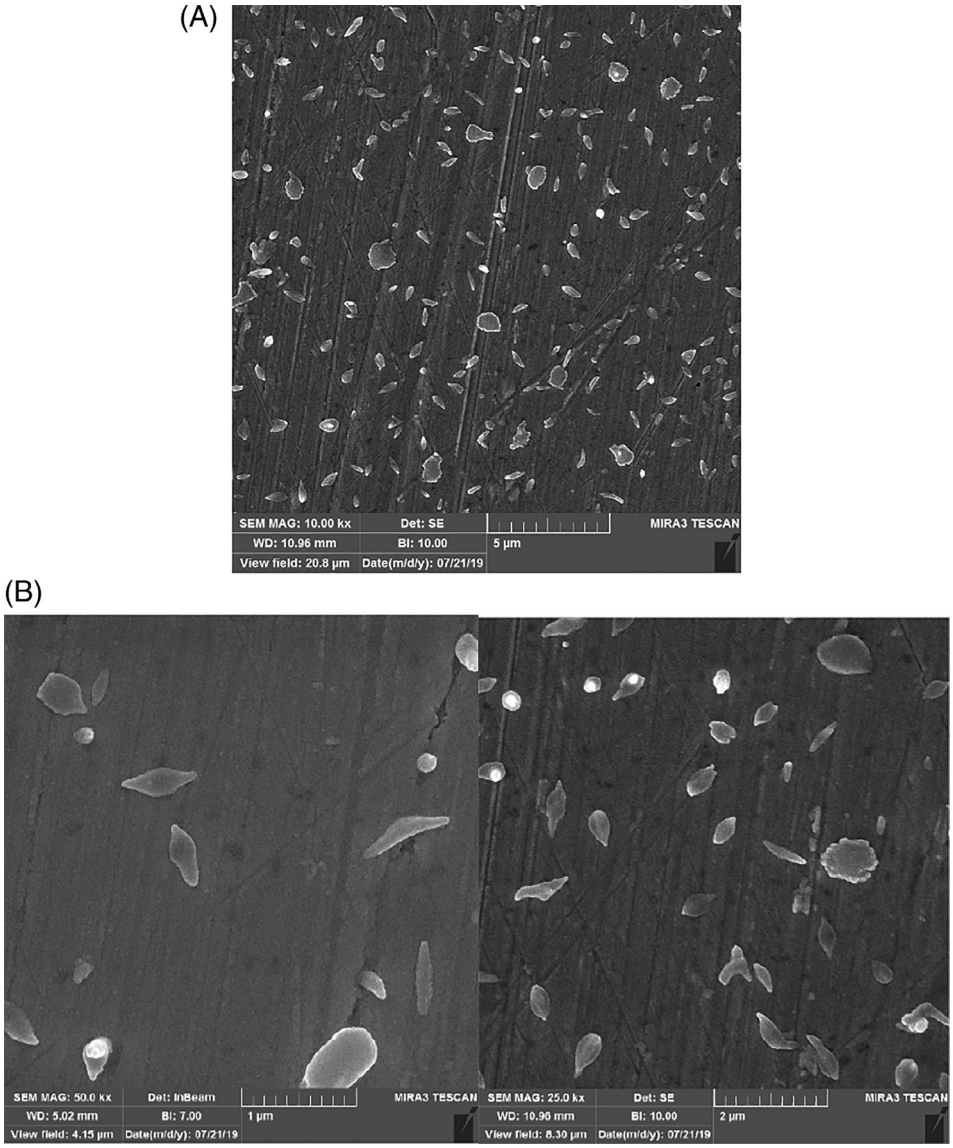


FIGURE 2 FE-SEM images of AuNPs-Au electrode in different magnification

Energy dispersive X-ray spectroscopy (EDS) is an analytical method used to analyze a sample's structural or chemical properties. The method relies on investigating the interaction between an X-ray excitation source and a sample.[22] The descriptive capabilities of this method are generally based on the general principle that each element has a unique atomic structure which enables a unique set of peaks in its X-ray spectrum. With this method, the sample surface is bombarded with an electron beam inside a microscope, and by the impact of the electron radiation on the electrons belonging to the sample atoms, some of these electrons are removed.[23,24] Since atoms cannot be left vacant and must be in equilibrium, electrons from the higher atomic layers migrate to the vacancy. To do this, the electrons with higher energy layers must lose some of their energy to adapt to the new layer's energy level and be stable when the energy is emitted in X-rays.[25] Accordingly, the EDS technique was applied in this work. As displayed in (Figure S3A-C), all expected elements presented in different steps of interface preparation.

3.3 | Treatment of pDNA

After a successful modification of AuNPs, the next step is immobilization of the DNA probe on the surface of the modified electrode. It is important to point out that the probe has been modified with the thiol (SH) group. Therefore, we have to use dithiothreitol (DTT) for the pretreatment of pDNA. DTT is one of the most common compounds used to reduce the disulfide bonds of proteins and in most cases to prevent intramolecular and intermolecular disulfide bonds from forming between cysteine residues of proteins. But, even DTT may not reduce hidden disulfide bonds, consequently, the reduction of disulfide bonds is sometimes encouraged under denaturing conditions such as high temperatures, or in the presence of a strong denaturant component.[26,27] For this to work, 0.01 M DTT and 0.01 sodium acetate was dissolved in DW. Then, equal amounts of the solution were mixed with 0.01 M of the DNA probe. The obtained solution was kept at room temperature for 30 minutes. Consequently, the solution was added to

the surface of the modified Au electrode and incubated at 4°C for 6 hours. After incubation, the electrode was rinsed with DW and then MCE was used for blocking of nonspecific spaces.[28] Our goal is to block nonreacted AuNPs with the DNA probe. In other words, MCE significantly increased the take-up of the cDNA at the next stage. The complete steps of genosensor assembly illustrated in Scheme 1.

3.4 | Assessment of electrochemical behavior of the electrode

Another important part of the present study is monitoring of biosensor preparation steps by electrochemical analyses. For this purpose, CVs of bare Au and Au modified AuNPs were recorded (Figure S4). Also, SWV technique was used for the monitoring of genosensor preparation steps (Figure S5). SWVs and CVs were recorded in the solution containing 1 mM of $K_3Fe(CN)_6^{3/4-}$ in 0.1 M aqueous KCl. According to the results obtained, the electron transfers kinetics of $Fe(CN)_6^{3-/4-}$ was disturbed. As shown in Figure S4, the stepwise assembly of AuNPs on the Au electrode is accompanied by a decline in the peak current of the electrodes. This occurrence is related to the enhanced electron transfer barriers presented upon the assembly of AuNPs.[29,30] In general, the results show that coating of AuNPs and immobilization of the pDNA were performed successfully.

Similar results were obtained by SWV technique (Figure S5).

3.5 | Optimization of TB incubation time

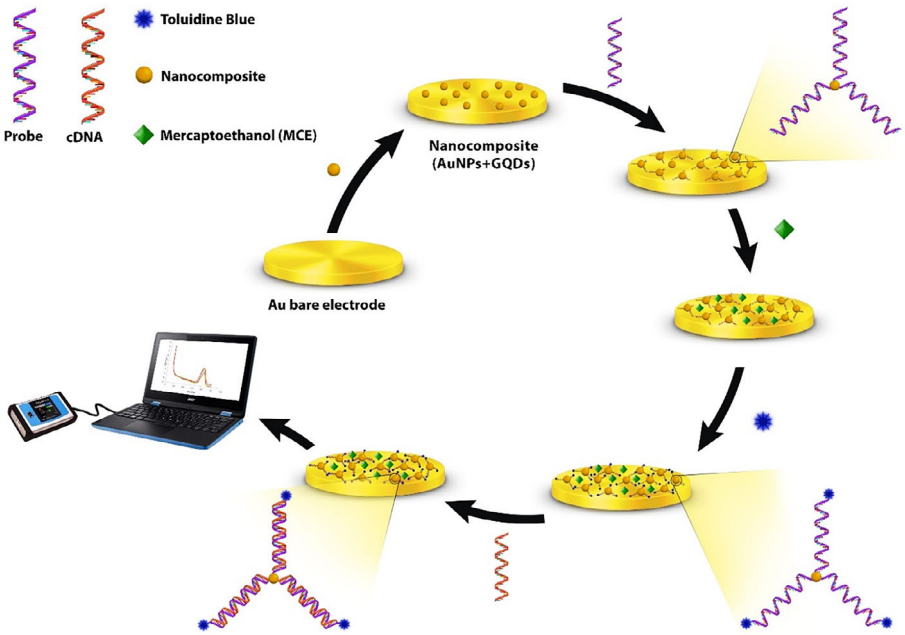
The application of hybridization redox indicators, allowed binding both to single stran DNA (ssDNA) and double strand DNA (dsDNAs) which permits the development of reusable biosensors. Also, the use of indicators eliminates a cumbersome and costly step of chemical conjugation of the redox-label for modification of the DNA strand. Toluidine blue (TB) is the frequently used redox indicator in biosensor technology and was applied for optimization of hybridization in this work. For this purpose, prepared genosensors were used in the TB solution (1 mg of TB in 10 mL of DW) for different times of 2, 5, and 10 minutes. According to Figure S6, maximum peak current was obtained at 5 minutes, and that means, oxidative reaction occurred after 5 minutes incubation at 37°C. Therefore, the optimum time for the incubation of the biosensor with TB was 5 minutes.

3.6 | Optimization of hybridization time

Hybridization of the immobilized probe to the surface of the Au modified electrode with the cDNA sequence is the basic part of the work of a genosensor preparation. Subsequently, finding optimal hybridization times is an essential principle in the development of a genosensor. For this purpose, 10 μL of cDNA was applied on the surface of the genosensor and incubated for different times: 15, 20, 30, 40, and 50 minutes. According Figure S7, maximum peak current was recorded at 30 minutes, and that means, optimum hybridization occurred after 30 minutes incubation at 37°C. So, optimum time for the hybridization of pDNA with cDNA was 30 minutes.

3.7 | Analytical approach

Hybridization with complementary DNA (cDNA) is the fundamental part of the genosensor performance.[31-33] In other words, the sensitivity of the system, which is the most important feature of a biosensor, is determined at this stage.[31,32] So, a different concentration of cDNA (10^{-6}, 10^{-9}, 10^{-12}, 10^{-15}, 10^{-17}, and 10^{-21} M) was prepared and immobilized on the surface of the biosensor. Figure 3 shows the peaks



SCHEME 1 The schematic illustration of the electrochemical genosensor for the detection of *Leishmania* spp

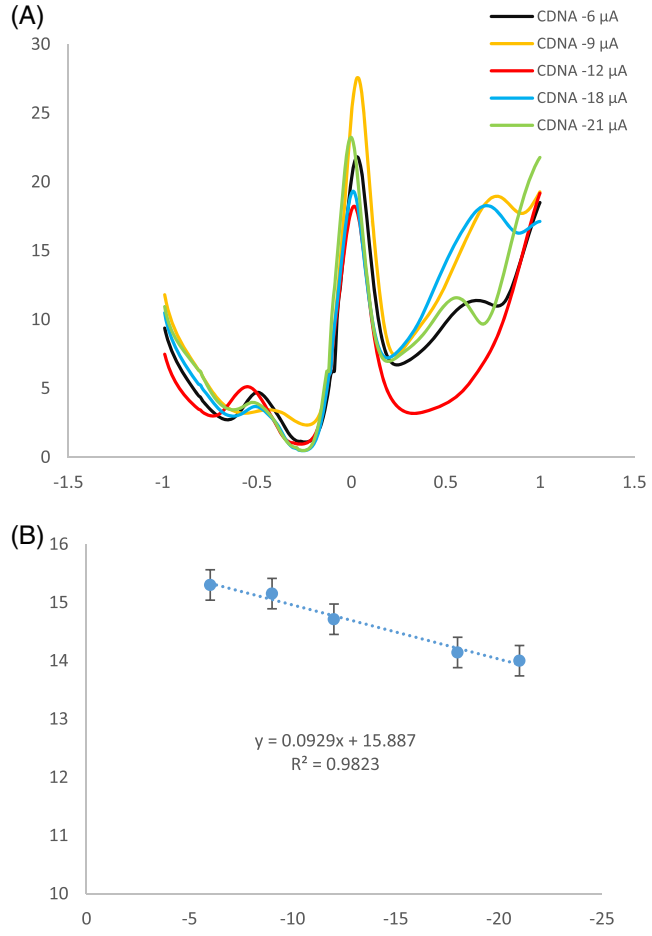


FIGURE 3 A, SWVs of the engineered biosensor for hybridization by cDNA in different concentrations (scan rate is 100 mV s^{-1}; TB = 20 μM; pH 7.4; pDNA = 100 μM). B, Calibration curve

of the cDNAs and their downward trend. In the other words, the engineered biosensor was able to detect a low amount (10^{-21} M) of target cDNA.

According to Figure 4, after hybridization of 5′-SH-$(CH_2)_6$ ATCTCGTAAGCAGATCGCTGTGTCAC-3′ with complementary target sequence (5′-GTGACACAGCGATCTGCTTACGAGAT-3′, the maximum peak current was appeared at 15.2 μA for 1 μM of cDNA. Interestingly, there is a linear relationship between peak current and concentration of cDNA. As can be seen in Figure 3, by reducting cDNA concentration to 1 ZM, the peak current decreased to 14 μA and regression equation was obtained as I (μA) = $0.0929C_{cDNA}$ + 15.887. These results confirmed that the genosensor is sensitive to detection of cDNA sequences in low concentrations (1 ZM). Also, a wide dynamic range (1 ZM to 1 μM) was obtained by this biosensor.

The appropriate limit of quantification can be considered as a result of several factors involved in the preparation of the desired genosensor, including excellent electrical conductivity, structural properties and high stability of the AuNPs, appropriate surface area which with the feasibility of biological activity was used to identify hybridization of pDNA with cDNA.

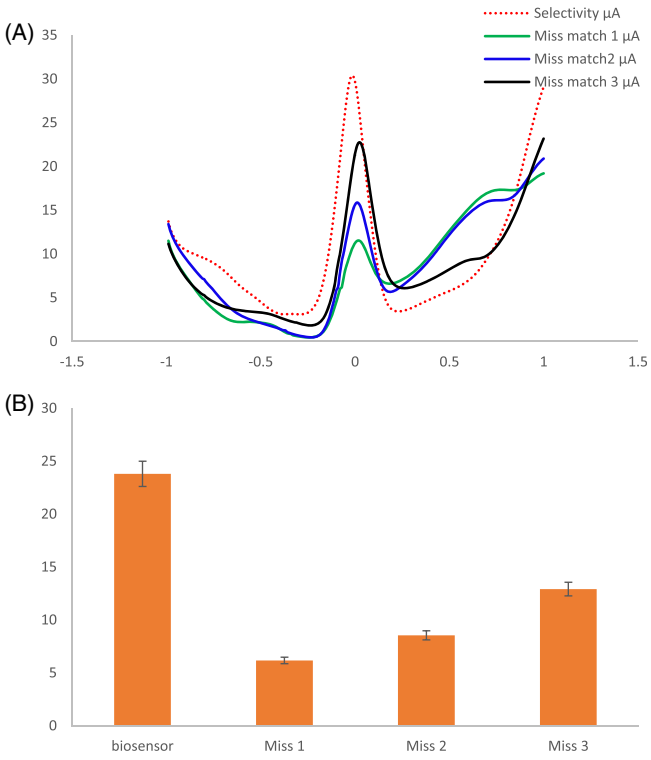


FIGURE 4 A, SWVs of designed biosensor in the presence of mismatch sequence of *Leishmania* spp (scan rate is 100 mV s^{-1}; TB = 20 μM; pH 7.4; pDNA = 100 μM). B, Histogram of peak currents vs type of sequences

In addition, AuNPs have double role in this biosensor. The terminal sulfur atoms of thiolated DNA has a bond to the AuNPs which lead to stable interaction of S—Au. Also, AuNPs led to signal amplification which is necessary for the sensitivity of the genosensor. Therefore, by using the engineered genosensor, excellent hybridization occurred and the appropriate peak current and LLOQ were obtained.

3.8 | Real sample analyses

Good performance in real environments such as body fluids including plasma, blood, cerebrospinal fluid, and urine are prominent features of an ideal biosensor. In this study, the sensitivity of the biosensors in humane plasma samples was also evaluated. For this purpose, the SWV technique was performed in supporting 2 mM plasma along with ferrocyanide/ferrocyanide as a supporting electrolyte. The results show that the proposed DNA-based method was able to detect in human plasma samples (Figure S8).

3.9 | Selectivity study

High selectivity is one of the essential features of an ideal gene-sensor. Selectivity analysis is measured using very similar sequences in hybridization and compared with specific sequence hybridization. In order to evaluate the selectivity of the proposed genosensor, three

mismatched sequences were applied respectively. 10 μL of the mismatch sequences were added on the surface of the genosensor and incubated for 30 minutes. The results are presented in Figure 4, and indicated that for three samples, the peak currents were changed slightly, compared to the biosensor. This indicates the high selectivity of the proposed biosensor which can discriminate the *Leishmania* genome from other similar sequences.

According to Figure 4, after hybridization of 5′-SH-$(CH_2)_6$ ATCTCGTAAGCAGATCGCTGTGTCAC-3′ with complementary target sequence (5′-GTGACACAGCGATCTGCTTACGAGAT-3′, the peak current was appeared at 24 μA. But by using a single nucleotide mismatch target sequence; 5′-CTGACACAGCGATCTGCTTACGAGAT-3′, a two nucleotide mismatch target sequence 5′-CCGACACAGCGAT CTGCTTACGAGAT-3′, and a three mismatch target sequence 5′-CCGACACAGCGATCTGCTTACGAGAT 3′) peak currents appeared at 5, 9, and 14 μA, respectively. These results confirmed that, the engineered biosensor is sensitive to the detection of the target sequence of *L pneumophila*.

3.10 | Regeneration

In order to calculate the regeneration study of the designed genosensor, after its hybridization with 10 μL of cDNA, it was immersed in hot water (50°C) for 3 minutes to de-hybridize. In the next step, the biosensor was re-hybridized with the same cDNA. As shown in Figure S9, the SWVs show slight changes occurred in the peak currents. So, regeneration of the genosensor was acceptable. As a result, the designed system can be reused.

Finally, it is important to point out that, mussel-inspired chemistry is an important method for surface modification and fabrication of functional materials.[34-45] In future, their applications especially in biomedical analysis should be cited.

4 | CONCLUSION

This study revealed fascinating method for rapid, accurate diagnosis of *Leishmania* spp infections using thiolated probe immobilized on the surface of AuNPs on the gold electrode. The appropriate limit of quantification can be considered as a result of several factors involved in the preparation of the desired genosensor, including excellent electrical conductivity, structural properties and high stability of the AuNPs, appropriate surface area which providing the feasibility of biological activity was used to identify hybridization of pDNA with cDNA. In addition, AuNPs have double role in this biosensor. The terminal sulfur atoms of thiolated DNA has a bond to the AuNPs which lead to stable interaction of S—Au. Also, AuNPs led to signal amplification which is necessary for increment of the genosensor sensitivity. Therefore, using the engineered genosensor, excellent hybridization occurred and appropriated peak current and LLOQ were obtained.

The most important feature of this biosensor is its high sensitivity (LLOQ = 1 ZM) and suitable linear range (10^{-6} to 10^{-21} M), promising to develop inexpensive and simple biosensors for the detection of *Leishmania*. Also, engineered genosensor is applicable in human plasma samples which is usable for clinical and environmental bio-analysis.

ACKNOWLEDGEMENT

This research work was supported by Tabriz University of Medical Sciences.

CONFLICT OF INTEREST

All the authors (A. Mobed, P. Mehri, M. Hasanzadeh, A. Mokhtarzadeh) have read, approved and made substantial contributions for the manuscript. None of the original material contained in this manuscript has been previously published nor is currently under review for publication elsewhere. Besides, the authors declare no conflict of interests and/or commercial products or companies.

AUTHOR CONTRIBUTIONS

Ahmad Mobed and Parina Mehri are co-first authors with equal contribution.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Mobed A, Mehri P, Hasanzadeh M, Mokhtarzadeh A. Binding of *Leishmania spp* with gold nanoparticles supported polyethylene glycol and its application for the sensitive detection of infectious photogenes in human plasma samples: A novel biosensor. *J Mol Recognit*. 2020;e2839. https://doi.org/10.1002/jmr.2839