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Research article

An innovative genosensor for the monitoring of *Leishmania spp* sequence using binding of pDNA to cDNA based on Cit-AgNPs

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ABSTRACT

Leishmaniasis considered as the most crucial epidemic-prone diseases according to the World Health Organization. Early diagnoses and therapy of *Leishmania* infection is a great challenge since, it has no symptom and is resistance to drugs. Therefore, there is an urgent need for sensitive and precise detection of this pathogen. In this study, a new method was developed for optical biosensing of *Leishmania spp* sequence based on hybridization of Citrate capped Ag nanoparticles bonded to specific single stranded DNA probe of *Leishmania spp*. Aggregation of the Citrate capped Ag nanoparticles in the existence or lack of a cDNA sequence of *Leishmania*, cause eye catching and considerable significant alter in the UV–vis. The obtained low limit of quantification (LLOQ) of was achieved as 1ZM. Based on experimental results in optimum conditions, quick bioanalysis of *Leishmania spp* sequence was performed (2 min). So, this probe can be used for the clinical diagnosis of this pathogen and infection disease.

1. Introduction

Leishmania is a unicellular eukaryotes with defined nucleus, kinetoplasts and flagella. They consist of two variable structure (Amastigote or Promastigote) depending on their lifecycle. Amastigote species are exists in blood circulatory systems of humans as well as mononuclear phagocytes [1, 2]. However, Promastigote species are exists in alimentary tract of sandflies. 21 species of this trypanosomes are able to cause illness in humans [3]. Moreover, approximately 6 million people in 98 countries are under the influence of it and virtually 0.9–1.6 million new cases are added to statistics annually. Leishmaniases are life treating diseases caused by more than 20 *Leishmania* species. Detection and analysing of this pathogen is vital since it reveals no symptom [4]. Self-rehabilitating cutaneous ulcer, mutilating mucocutaneous and lethal systemic malfunction are some signs of *Leishmania* infection [5]. This pathogen can conceal in phagocytotic cells like neutrophils and macrophages, hence, escape from immune system destruction. Moreover, enzymes which digest the pathogen are not able to detect and destroy them, therefore, it will propagate very rapidly, suppress macrophage and immune system and leads to cell apoptosis [2]. According to the World Health Organization, *Leishmaniasis* is considered as the most crucial epidemic-prone diseases. So, there is an urgent need for the sensitive and precise detection of this pathogen [6]. Multiple conventional methods have been used for this purpose, like culture [7], microscopy [8] and molecular methods [9]. However, tradition methods are time-consuming and laborious since requiring lots of samples with limited sensitivity. Moreover, more sophisticated and sensitive met strategies are required for identification of *Leishmania spp*. Aggregation method offer promise for tackling drawbacks of conventional techniques, supply a set of promising methodologies for large scale, fast and accurate investigations. During this method, clusters are formed because of particle transports and interfacial chemical mechanisms in aqueous media. This processes are usually fractal in nature [10, 11]. If the aggregation procedure were low, particles have more time to configure themselves. Hence, denser structures will be created. This physical structure and density of the aggregates affect the reactive surface area, reactivity and bioavailability [11] (see Table 1).

Moreover, aggregation of nanoparticles leads to variations in absorption spectra and significant colour changes of solutions. In the presence of analytes the aggregation of silver nanoparticles (AgNPs) will occur and lead to alternation in solution colour. Therefore, this phenomena can be applied for chemical and biological sensors developments in order to detect multiple compounds [12].

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Table 1. Comparison of previously developed methods for determination of *Leishmania spp.*

Type of Pathogen	Method	Nanoparticeles	Detection range	Detection Limit	Year/Ref.
Leishmania major	spectrophotometric	gold nanoparticles	1 μM-1ZM	1ZM (LLOQ)	2016 [17]
Leishmania	UV–vis spectrophotometry and electrochemical impedance spectroscopy	AuNP/CdS/ITO	1 up to 300 nmol L^{-1}	0.41 nmol L^{-1}	2018 [18]
Leishmania	surface plasmon resonance (SPR)	-	10 $mg.mL^{-1}$ to 50 $mg.mL^{-1}$	-	2013 [19]
Leishmania donovani	Spectroscopy and Scanning Electron Microscopy	indium tin oxide (ITO)	2 pg ml^{-1} to 2 μg ml^{-1}	2 fg ml^{-1}	2011 [20]
Leishmania	electrical impedance spectroscopy	thiol modified CNTs	0.1 to 98.3 fg/μL	0.1 fg/μL (15 fM)	2017 [21]
Leishmania	cyclic voltammetry (CV) and electrochemical impedance spectroscopy	gold nanoparticles (PANIAuNp)	-	0.01 pg mL^{-1}	2016 [22]
Leishmania	Immunosensor/SPR	gold nanoparticles	9.70–51.8 nmol L^{-1}	7.37 nmol L^{-1}	2015 [23]
Leishmania Amazonensis	Immunosensor	gold nanoparticles	1×10^{-5} mg mL^{-1}	10-5 mg mL^{-1}	2010 [23]
Leishmania	UV/Vis spectroscopy and fluorescence microscopy	Cit-Ag Nano particles	1nM-1ZM	1ZM (LLOQ)	This work

Research work toward the development of optical biosensor based on the phenomenon of Citrate based AgNPs aggregation has been performed, In order to detect *Leishmania* single stranded DNA.

The importance of silver nanoparticles (AgNPs) for developing colorimetric and optical biosensors are undeniable due to their privileged optical and extinction coefficient potentials. Moreover, these materials provide suitable response to detection of some range of targets like drug, ion, protein, pathogen, and *etc*. [13].

In this study citrate capped silver nanoparticles (Cit-AgNPs) was synthesized and characterized as a suitable probe to develop an innovative method for optical determination of *Leishmania* based on UV/Vis and fluorescence.

Spectroscopic methods are most frequently used for analysing compounds since they provide rapid and simple procedure. Maxima and minima spectra of analytes in spectrophotometry and spectrofluorometery are considerably vital for qualitative analysing of different targets. Moreover, spectroscopic analyses had occurred to conform the deference between spectra of bare DNA probe of *Leishmania* and hybridized pDNA with Citrate capped Ag nanoparticles [14].

In this study we took advantage of Citrate-Ag nanoparticles aggregation in aqueous media in order to detect specific single stranded DNA probe. On the other hand it has been found that cooperative functionalities of citrate compounds strongly promote the aggregation of AgNPs. Aggregation of the Citrate capped Ag nanoparticles in the existence or lack of a cDNA sequence of *Leishmania*, cause eye catching and considerable significant alter in the UV–vis. The obtained low limit of quantification (LLOQ) of was achieved as 1ZM. Based on experimental results in optimum conditions, quick bioanalysis of *Leishmania* spp was performed (2 min). So, this probe can be used for the clinical diagnosis of this pathogen and infection disease.

2. Experimental

This part is similar to our previous works [15, 17].

3. Results and discussion

3.1. Leishmania primer (p DNA) activating

Leishmania primer was activated by adding Dithiotrietol (DTT) to pDNA sequences encounter in microbuses [16].

Thiolated end of *Leishmania* primer could be reduced or deprotected by DTT. Also, Thiolated pDNA could form dimer via their sulfur atoms when oxygen is peasant. Furthermore, DTT act as protecting agent through preventing oxidation of thiol groups, 0.01 M of DTT and 0.01M of sodium acetate (10 ml) was utilized to produce DTT solution in deionized water. Then, 15 μl of pDNA was added to 10 μl of the prepared solution and incubated for 15 min. In the following step, 200 μl of ethyl acetate was mixed with prepared solution and vortexed for 5 min and whole solution centrifuged for 10 min in 8000 rpm. Supernatant was discarded and mixed with 200 μl of ethyl acetate and centrifuged again for 10 min in 8000 rpm. As usual supernatant discarded and 200 μl of AgNPs added to solution and incubated for 2 h in 45 °C. After 2 h, 400 μl of AgNPs mixed with pDNA poured in the cuvettes and spectroscopic analysis were recorded.

3.2. Time optimization of Leishmania cDNA hybridization with pDNA

Hybridization of DNA probes with complementary sequences was occurred in different time intervals. For this purpose, 15 μl of cDNA was

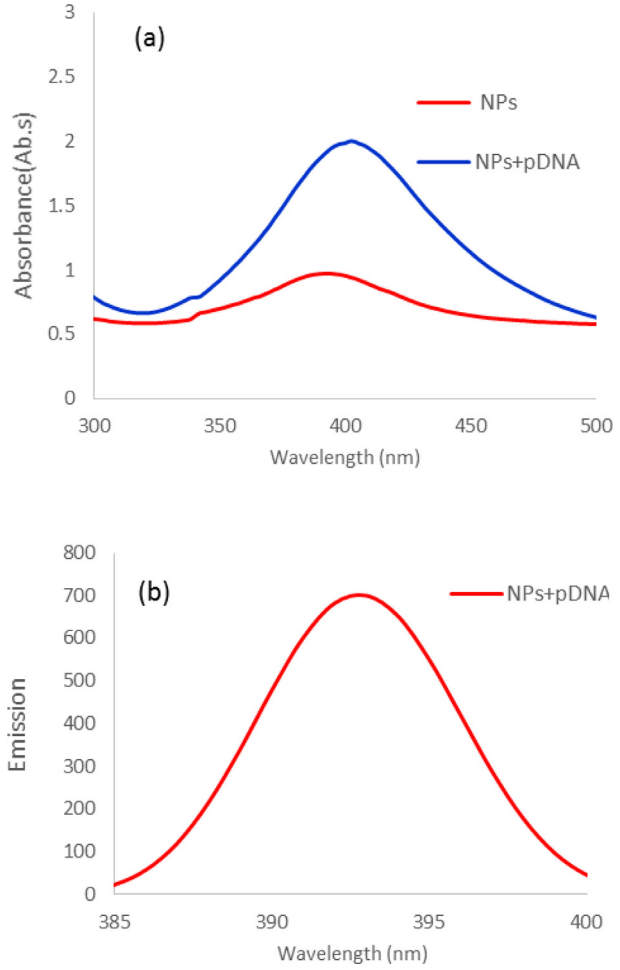

Figure 1. **(a)** Uv/Vis absorbance spectrum of Citrate-AgNPs and Citrate AgNPs after counjugation with pDNA **(b)** Fluorescence emission spectrum of Citrate AgNPs after counjugation with pDNA.

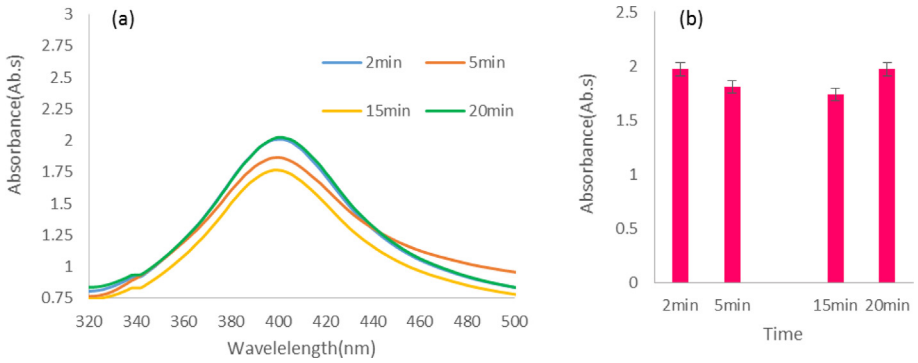

Figure 2. **(a)** Uv/Vis absorbance spectrum of Citrate-AgNPs after conjugation with pDNA in different incubation time (2, 5, 10,15 and 20 min). **(b)** Histogram peak intensity of a Citrate-AgNPs after conjugation with p DNA in different incubation time (2, 5, 15 and 20 min).

mixed with *Leishmania* pDNA (15 μl) then 200 μl of Citrate-AgNPs were mixed with solution and incubated for 2h in 45 °C. In order to optimize reaction procedure, 1 mM of NaCl was mixed with solution and UV/Vis spectra with fluorescence spectra of prepared solutions were recorded successively in 2,5,10,15, and 20 min, finally.

3.3. Optimization of different concentrations of Leishmania cDNA hybridized with pDNA

Different concentrations of cDNA (10^{-9}M, 10^{-12}M, 10^{-17}M and 10^{-21}M) were made (Similar to previous step). 10 ml of 0.01M sodium acetate were mixed with 0.01 M of DTT in deionized water (DW) and 15 μl of pDNA was added to 10 μl of prepared solution which and incubated for 15 min. Then, the solution mixed with 200 μl of ethyl acetate and shake for 5 min like previous step, all the solutions centrifuged for 10 min in 8000 rpm. This step repeated twice, then, supernatant was removed and, AgNPs (200 μl of) were mixed and incubated for 2 h in 45 °C. Finally, in order to increase the stability of silver nanoparticles 15 μL of NaCl (1 mM) was mixed with solution and incubated for 2 min.

3.4. Selectivity of Leishmania cDNA hybridization with pDNA

In this part of study, some of mismatch sequences of *Leishmania spp* probe [17] were used to evaluate as negative control. Using this approach, we are able to test selectivity of target sequence by developed method. Finally, mismatch primers (15 μl) were added to *Leishmania* pDNA and incubated untie conducting spectroscopic tests and recording analytical data using UV/Vis and fluorescence methods.

3.5. Stability of Leishmania cDNA hybridization with pDNA

Tests were repeated at three different days and 3 times in one day to evaluate the intra-day and intro-day stability of *Leishmania* cDNA hybridization with pDNA. Introduced platform was stable for 24 h and useful totally.

4. Analytical study

As displayed in Figure 1 (a,b) a considerable change revealed in UV-Vis spectrum and fluorescence spectra of solutions in the present of

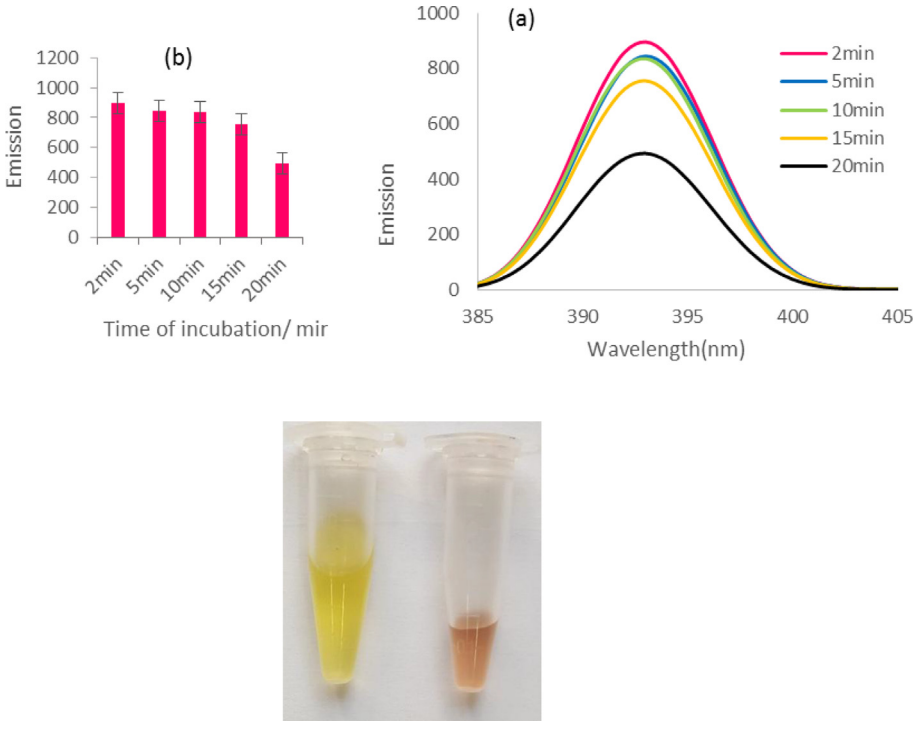

Figure 3. **(a)** Fluorescence spectra of Citrate-AgNPs after conjugation with pDNA in different incubation time (2,5,10, and 15 min). **(b)** Histogram peak intensity of Citrate-AgNPs after conjugation with p DNA in different incubation time (2,5,10,15, and 20 min) (n = 3, SD = 1.25). **(c)** Color change befor and after onjugation.

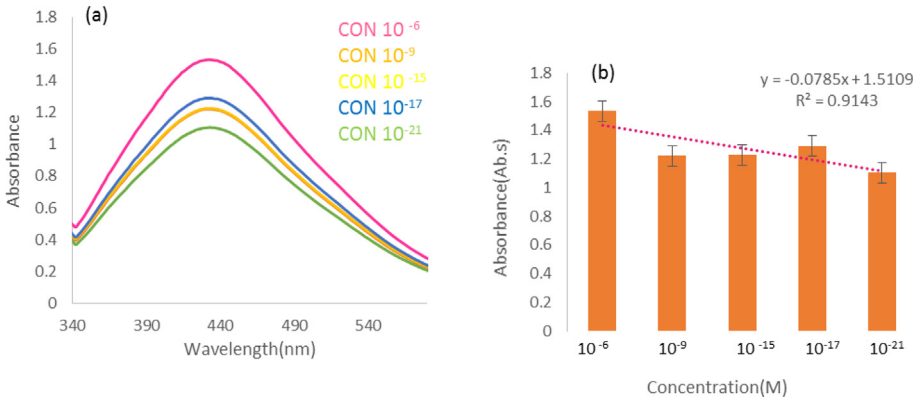

Figure 4. (a) Uv/Vis absorbance spectrum of hybridization of *Leishmania* cDNA with pDNA in various concentrations (10^{-6},10^{-9},10^{-12}, 10^{-17} and 10^{-21}M). **(b),** Calibration curve.

pDNA. It display covalent bonding of citrate capped silver nanoparticles to the thiol groups of probe oligonucleotides. According to spectrophotometric data Citrate capped Ag NPs have wavelength of 390 nm with intensity of approximately 1.93. However, in the present of pDNA, UV-Vis spectrum peak appeared in the same wavelength with the intensity of 2. Moreover, fluorescence spectrum peak revealed in 393 nm with intensity of 700. Obtained, results display that, covalent bonding of citrate capped silver nanoparticles to thiol groups of *Leishmania* primer enhances fluorescence emission spectra peak intensity.

4.1. Analytical approaches of time optimization of Leishmania cDNA hybridization with pDNA

UV/Vis spectra and fluorescence spectra of DNA probes hybridization with *Leishmania* complementary sequences were recorded at various successive times (2, 5, 10,15 and 20, min). Figure 2 indicates that, UV/Vis peak of Citrate capped Ag NPs with pDNA is at wavelength of 402 nm with intensity of 2.011, 1.862, 2.112, 1.76, 2.025 in 2, 5, 10, 15, and 20 min respectively.

The fluorescence spectrum peak of Citrate capped Ag NPs bonded to pDNA appeared at wavelength of approximately 393 nm with intensity of 896.5, 844.9, 835.73, 757.75, and 493.23 in 2, 5, 10, 15, and 20 min, respectively. It seems that the best reaction will occur after 2 min. Therefore, the optimization hybridization time of *Leishmania* cDNA with pDNA is 2 min (Figure 3).

4.2. Analytical evaluation of biosensor performace

UV-Vis spectrum peak of Citrate capped AgNPs with pDNA in different concentrations of cDNA (10^{-9}M, 10^{-12}M, 10^{-17}M and 10^{-21}M) were recorded.

Figure 4 indicates that, the UV-Vis spectrum peak of Citrate capped AgNPs with pDNA appeared at wavelength of 402 nm with intensity of 1.532, 1.222, 1.227, 1.291, 1.105 for the concentration of 10^{-6}, 10^{-9}, 10^{-15}, 10^{-17}, and 10^{-21} M, respectively.

Results indicated that, the designed biosensor could detect 1ZM of target sequence (cDNA) with dynamic range of 1 nM- 1ZM. Citrate-Ag NPs with pDNA exibated the fluorescence spectrum peak at wavelength of approximately 402 nm with intensity of 848.76, 843.43, 899.42, 924.27 for the concentration of 10^{-6}, 10^{-9}, 10^{-12}, 10^{-15}, 10^{-17}, and 10^{-21} M, respectively. Moreover, results indicated that, although the trend of the recorded signals were downward, it was able to detect cDNA on the low concentration of 1ZM and dynamic range of 1 nM- 1ZM. Based on the obtained results, there is a linear relation between peak intensity of UV/Vis results, log $C_{pathogen}$

4.3. Selectivity study

For assessment selectivity of *leishmania* cDNA hybridization with pDNA, 3 mismatche sequences (5′CTGACACAGCGATCTGCTTACGAGAT 3′) GC ratio:50% Tm:63.7 basecount:26 ،(5′CCGACACAGCGAT CTGCTTACGAGAT 3′) GC ratio:53.9% Tm:65.6 ،basecount:26 and (5′CCGACACAGCGATCTGCTTACGAGAT 3′) GC ratio:53.9% Tm:65.6 ،basecount:26 were utilize. Spectroscopic tests were conducted and analytical approaches were recorded. According to Figure 5, the UV-Vis spectrum peak of Citrate-Ag NPs with p DNA and 3 different mismatches, was at wavelength of 438 nm with intensity of 1.35, 1.28, 1.1 for mismatch 1, mismatch 2, and mismatch 3 at wavelength of 402 nm with

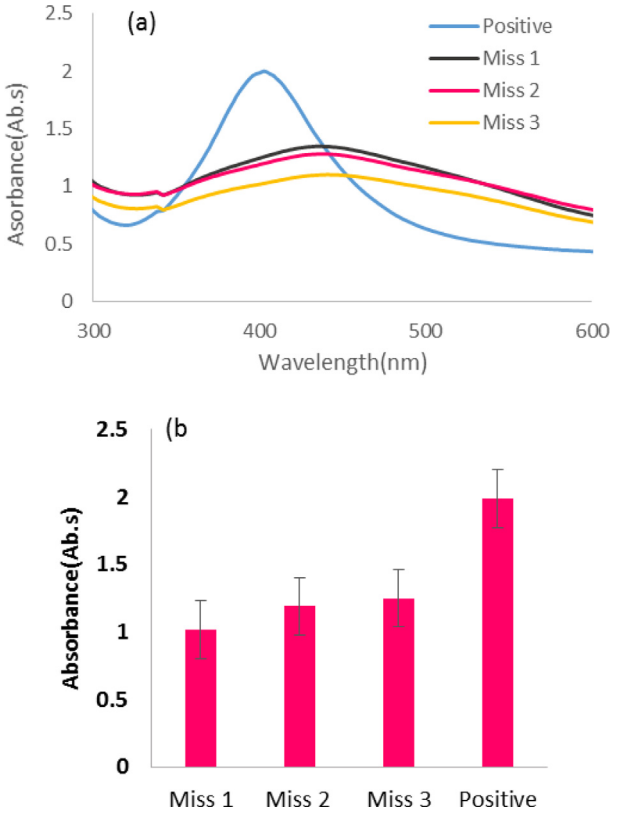

Figure 5. (a) Uv/Vis absorbance spectrum of cDNA hybridization with missmatch 1, mismatch 2 and missmatch 3. **(b)** Histogram of cDNA hybridization with mismatch 1(5′CCGACACAGCGATCTGCTTACGAGAT 3′), mismatch 2(5′CCGACACAGCGATCTGCTTACGAGAT 3′) and missmatch 3(5′GTGACACA GCGATCTGCTTACGAGAT 3′) and positive sequences.

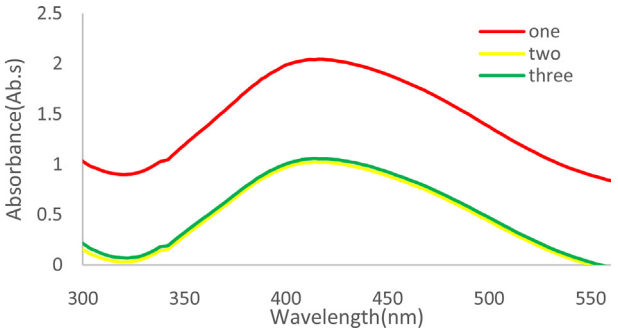

Figure 6. **(a)** Fluorescence and emission spectrum of designed biosensor in 24h of storage. **(b)** UV/Vis absorbance spectrum of designed biosensor in 24h of storage.

intensity of 2 for positive samples respectively. Obtained results indicate that, for three negative control sequences, the peak intensity position were changed, compared to the biosensor. So excellent, the proposed highly selective optical biosensor can detect *Leishmania* genome from other similar sequences accurately.

4.4. Evaluation of stability of Leishmania cDNA hybridization with pDNA

Being stable is one of the basic characteristics of ideal biosensor. Cit-AgNPs have been used in this study exhibited well stability in this study. Silver nanoparticles have wide applications in the treatment of parasite infections due to their specific chemical and physical properties. Stability tests was measured at 3 different times in a day to evaluate the stability of *Leishmania* cDNA hybridization with pDNA. As shown in Figure 6 (a,b), the created platform is stable for 24 h completely and useable totally.

The reproducibility of the genosensor was evaluated by preparation of four conjugated solution at the same condition, and using them for detection of 1 nM to 1zM of Leishmania *spp* genome the relative standard deviations were 1.22% and 2.33% for detection of 1 nM to 1zM of *Leishmania spp*, respectively. These results introduce reliable platform and acceptable precision of the immunosensor.

It is important to point out that, there is other optical probes which has efficient activity for the detection of *Leishmania spp*. Therefore, researchers can be test these nanomaterials/probes for the detection and sensitive determination of some pathogens [24, 25, 26, 27, 28].

5. Conclusion

In this work, biosensing *Leishmania spp* using novel optical probe (citrate capped silver nano particles) was conducted. Based on optical properties of this nano-prob, aggregation of Cit-AgNPs cause variations in absorption spectra and significant colour changes in solutions. Hence, in the presence of analytes the aggregation of this nanoparticles can occur and alternation in solution colour will be caused. We took advantage of this phenomenon for developing an innovative biosensor in order to detect *Leishmania* single strand DNA. We utilized citrate capped silver nanoparticles as powerful optical probe with high affinity binding due to its citrate molecules to *Leishmania* pDNA sequences. So, aggregation of nano-probe in the presence of target cDNA was occurred and spectroscopic analyses was performed. Using proposed method, *Leishmania* spp was detected in low concentration (1 ZM) with short analysis time (2 min). Finally, evaluation of the selectivity of propose method was tested in the existence of some mismatch probes. Overall, the present study paved the way for quick (2 min) and accrued recognition of *Leishmania* spp, which can be good alternative method to the traditional techniques to clinical diagnosis of infectious disease.

Declarations

Author contribution statement

Parina Mehri, Paria Pashazadeh-Panahi: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Mohammad Hasanzadeh: Conceived and designed the experiments; Analyzed and interpreted the data.

Nasrin Razmi: Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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