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Development of direct assays for *Toxoplasma gondii* and its use in genomic DNA sample

Lívia M. Alves[a], Vinícius R. Rodovalho[a], Ana C. H. Castro[a], Márcia A. R. Freitas[a] Caroline M. Mota[b], Tiago W. P. Mineo[b], José R. Mineo[b], João M. Madurro[c], Ana G. Brito-Madurro[a]*

[a]Institute of Genetics and Biochemistry, Federal University of Uberlândia, Uberlândia, Brazil

[b]Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, Brazil

[c]Institute of Chemistry, Federal University of Uberlândia, Uberlândia, Brazil

*Author to whom correspondence should be addressed. Tel. +55 34 32182203; Fax: +55 3432182203; E-mail: agbrito@ufu.br

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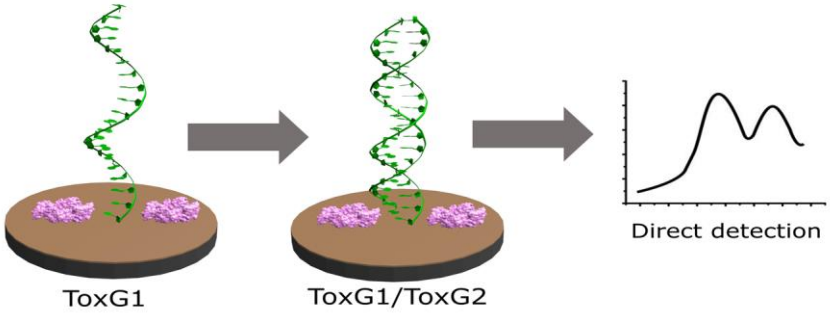
GRAPHICAL ABSTRACT



Abstract

HIGHLIGHTS

A new biosensor to toxoplasmosis was developed based on modified electrode;

The biosensor was able of detect until 100 ng.mL^{-1} of the *T. gondii* genomic DNA;

The genosensor showed high selectivity, discriminating non-specific targets;

Optical assays showed significant change in the absorbance peak in presence of *Toxoplasma gondii* genomic DNA.

This work describes an approach for the selection and detection of specific DNA probes related to *Toxoplasma gondii*, a protozoan parasite responsible for toxoplasmosis. The detection system was developed on graphite carbon electrode modified with poly(3-hydroxybenzoic acid) sensitized with ToxG1 probe. The hybridization of the specific genomic DNA related to *T. gondii* showed good response by direct detection of guanine residue oxidation using differential pulse voltammetry (DPV). The biosensor was able to distinguish both the complementary and non-complementary targets and detect up to 100 ng.µL^{-1} of the *T. gondii* genomic DNA. The hybridization (ToxG1: *T. gondii* genomic DNA) was confirmed by optical measurement. Optical assays using gold nanoparticles:ToxG1 probe showed a significant change in the absorbance peak in the presence of the *T. gondii* genomic DNA according to the electrochemical results. This novel biosensor shows potential as electrochemical transducer and was successfully applied in the biological sample.

Keywords: *Toxoplasma gondii;* DNA; GRA6 gene; biosensor; gold nanoparticles.

1. Introduction

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Toxoplasma gondii is the intracellular protozoan parasite responsible for toxoplasmosis, a zoonosis with medical and veterinary importance worldwide [1]. Most infections are asymptomatic in healthy adults, but severe disease may develop with congenital infections or immunodepression [2]. Estimates show that more than a third of the world's population has been infected with this parasite [3]. Diagnosis and genetic characterization of *T. gondii* infection are crucial for monitoring, prevention and control of toxoplasmosis. Traditional approaches for toxoplasmosis detection rely on immunological diagnosis and imaging techniques [4]. These methods have limitations; as for instance, the identification based on imaging techniques is less sensitive and uncertain. In addition, these methods demand a long time for sample preparation, expensive equipment and the need for skilled personnel.

There is a significant need for improved diagnosis. Biosensors stand out as a promising alternative to the traditional methodologies. Biosensors are analytical devices that combine the specificity of biomolecules with sensible transduction platforms, presenting portability, rapidity and good cost-effectiveness relation. In the literature, sensors developed for the detection of *T. gondii* are generally based on the immobilization of antibodies [5-7], aptamers [8] and DNA probes [9, 10] The detection of specific DNA sequences plays a major role in many fields, especially in clinical diagnostics. Direct monitoring of changes in its electrochemical properties, such as oxidation of guanine and adenine residues, enables the detection of hybridization on the electrode surface [11].

The combination of polymeric materials with biosensors promotes an increase in the analytical performance of the biosensor. Polymers derived from hydroxybenzoic acid are used in the development of polymeric films for the modification of electrodes due to the presence of functional groups (carboxylic acid and hydroxyl acid), which are liable to undergo electropolymerization and to interact with biomolecules [12].

Nucleic acid components, such as nucleosides and nucleotides, are electroactive species that can be monitored. The electrochemical behavior of DNA and the effects of its adsorption onto several types of electrodes have been widely investigated [13-15]. Oliveira-Brett and collaborators [11] investigated the voltammetric oxidation of all nucleotides of deoxyribonucleic acid on glassy carbon electrodes. Silva et al. [16] successfully described the immobilization of purine and pyrimidine bases on graphite electrodes modified with poly(4-methoxyphenethylamine).

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The focus of the current work was the development of a method for the selection of specific DNA probes related to *T. gondii* and a system for the detection of a specific target sequence for the diagnosis of toxoplasmosis based on graphite electrodes modified with poly(3-hydroxybenzoic acid) and sensitized with DNA-oligonucleotide probe (Fig. 1). In this work, it was possible to detect double-strand DNA related to *T. gondii* onto the modified surface. To the best of our knowledge, this is the first genosensor that uses specific genomic DNA as a target for the diagnosis of *T. gondii* infection or toxoplasmosis.

Here Fig. 1

2. Experimental

2.1. Chemicals

All reagents used were of analytical grade without previous purification. Ultra high purity water (Master System, Gehaka, Brazil) was used for the preparation of all solutions. The monomer employed for the polymerization reaction was 3-hydroxybenzoic acid (3-HBA) 99%, purchased from Sigma–Aldrich (USA). The detections were performed in 0.1 mol.L^{-1} phosphate buffer (Na_2HPO_4 0.061 mol.L^{-1}, NaH_2PO_4 0.039 mol.L^{-1}, pH = 7.4). All solutions were deoxygenated by ultra-pure nitrogen bubbling for at least 45 minutes before use. Experiments were conducted at room temperature (25 ± 1°C).

Oligonucleotides were synthesized by Invitrogen Life Technologies and their sequences are: ToxG1 (probe): 5'-CTGGGAACGGTGGGAATGAA-3', ToxG1S (probe) 5'-CTGGGAACGGTGGGAATGAA-SH-3', ToxG2 (target): 5'-TTCATTCCCACCGTTCCCAG-3', Non-complementary target: 5'-CCACTCTTCAATTCTCTCCGCC-3'.

The oligonucleotide stock solutions and dilutions were prepared in SSC buffer (pH 7.0, sodium chloride 0.3 mol.L^{-1} and sodium citrate 0.03 mol.L^{-1}, Sigma–Aldrich, USA) and stored at −12ºC.

2.2. Apparatus

The electrochemical studies were performed in a potentiostat from CH Instruments, model 620C, USA. A graphite disk (6.15 mm diameter, 99.9995% purity)

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from Alfa Aesar was used as working electrode. A platinum plate and silver/silver chloride (3.0 mol L^{-1}) were used as auxiliary and reference electrodes, respectively. Film morphology in the absence or presence of biomolecules was assessed by atomic force microscopy (SPM 9600, Shimadzu). The assays using Gold nanoparticles (Au NPs) were carried out using a spectrophotometer (UV-1650PC, Shimadzu).

2.3. In silico probe selection

Nucleotide sequences of GRA6 gene (GenBank: L33814.1), B1 gene (GenBank: AF179871.1) and a repetitive region (GenBank: AF146527.1) from *T. gondii* genome were obtained from the National Center for Biotechnology Information (NCBI) database. DNA probes were selected *in silico* using a pipeline that integrated Primer3 software [17] with algorithms for multi-fast handling and parameter setting, as well as scoring, sorting and formatting of the results.

The developed pipeline took fasta files as input, along with parameters specified in the main script, such as probe size, GC content, scoring scheme, DNA and salt concentrations, constraints of melting temperature for desirable probes and undesirable secondary structures. Afterwards, Primer3 parameter setting and execution were sequentially conducted. The results obtained were scored with a user-defined scheme that accounted for occurrence of pairs, triplets and quartets of specific base residues, since some of them are preferred for electrochemical direct monitoring in modified carbon electrodes (G and A) [11]. Next, the results were ordered according to the score and formatted for the generation of detailed output files. Finally, the probes obtained were submitted to BLAST alignment with the nucleotide database in order to select the most species-specific probes.

2.4. Parasite preparation and genomic DNA extraction

Strains of *Neospora caninum* (NcLivΔHPT) and *T. gondii* tachyzoites (RH) were maintained by serial passages in HeLa cell line (CCL-2, ATCC, Manassas, VA, USA), cultured in RPMI 1640 medium supplemented with 2 $mmol.L^{-1}$ glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37°C in 5% CO_2 atmosphere. Parasite suspensions were obtained as previously described [18]. Briefly, tachyzoites were harvested by scraping off the cell monolayer after 2-3 days of infection, passed through a 26-gauge needle and centrifuged at low speed (45 × g) for 1 min, for debris removal.

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The supernatant containing parasite suspension was collected, washed in phosphate buffered saline (PBS, pH 7.2) and centrifuged at 1,000 × g for 10 min. After the centrifugation, the pellet was kept at - 20 °C for DNA extraction.

The genomic DNA was extracted from NcLivΔHPT *N. caninum* and RH *T. gondii* tachyzoites. The parasites were lysed and digested with 200 µL lysis buffer (400 mmol.L^{-1} NaCl, 10 mmol.L^{-1} Tris-HCl, 2 mmol.L^{-1} and EDTA, pH 8.2), 6 µL 10% SDS, 3 µl proteinase K (20 mg.mL^{-1}), and incubated at 55°C overnight. After incubation, NaCl (150 µL, 6 mmol.L^{-1}) was added into the solution. The mixture was centrifuged at 12,000 × g at 4 °C for 10 min. The supernatant was transferred to the 1.5-mL tube, ethanol (800 µL, 100%) was added and centrifuged (12,000 × g for 10 min). The precipitated DNA was washed with ethanol (1 mL, 75%) and solubilized in 20 µL of Milli-Q water.

The human and mouse genomic DNA was extracted from peripheral blood according to Al-Shuhaib [19].

The concentration and quality of DNA extracted from each sample were analyzed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). After that, the DNA samples were stored at −20 °C until use.

2.5. Preparation of graphite electrode modified with poly (3-HBA)

The bare graphite electrodes were polished mechanically with alumina slurry (0.3 µm), sonicated, washed with deionized water and dried in air. The preconditioning was performed in 0.5 mol.L^{-1} $HClO_4$ solution through cyclic voltammetry between + 0.0 and +1.2V, 4 cycles, 50 mV.s^{-1}. The electrodes were modified with polymer derived from 3- hydroxybenzoic acid according to Ferreira and collaborators [12] with modifications. The electropolymerization was conducted on graphite electrodes (10 scans, 0.0 and +1.2 V *vs.* Ag/AgCl, 50 mV.s^{-1}) from a 3-HBA solution (2.5 mmol.L^{-1}) prepared in $HClO_4$ (0.5 mol.L^{-1}) at room temperature (25 ± 1ºC).

2.6. Effect of probe concentration in the immobilization and hybridization

Optimization studies of the probe concentration were performed by adding 10 µL of different concentrations of ToxG1 (5 µmol.L^{-1}, 15 µmol.L^{-1}, 20 µmol.L^{-1}, 25 µ mol.L^{-1}, 35 µmol.L^{-1} and 50 µmol.L^{-1}) in the modified electrode surface at 37ºC and incubated for 30 minutes. The blocking of non-specific binding was performed with

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BSA 1% (w/v) at 37ºC for 1 h. After immobilization of the oligonucleotide probes by physical adsorption, 10 µL of different concentrations of ToxG2 target (10 µmol.L^{-1}, 30 µmol.L^{-1}, 40 µmol.L^{-1}, 50 µmol.L^{-1}, 70 µmol.L^{-1} and 100 µmol.L^{-1}) were added onto the modified electrode surfaces.

For the tests using the genomic DNA, the samples were maintained at 98 °C for 5 minutes, in order to de-hybridize the strands; they were then added onto the modified electrodes surfaces and incubated for 15 minutes at 52°C.

After each modification, the electrodes were washed three times with 50 µL of phosphate buffer. Differential pulse voltammetry measurements were performed using phosphate buffer as electrolyte.

2.7. Assay using AuNPs

The AuNPs were produced using the modified Turkevich-Frens method. In this method, gold salt is reduced, occurring the nucleation and growth of gold nanoparticles of 15 to 20 nm, stabilized by citrate ions [20]. After synthesis, the AuNPs were stored at 4°C until the use. The AuNPs sensibilizated with DNA probe were preparated using 200 µL of AuNPs solution added to probe solution, ToxG1S (2 uL, 200 µmol.L^{-1}) and maintained at room temperature for 1 hour. Prior to the hybridization process, the genetic material was digested using the EcoRI restriction enzyme (2 U/L) diluted in the specific buffer (50 mmol.L^{-1} NaCl, 10 mmol.L^{-1} Tris-HCl, 10 mmol.L^{-1} $MgCl_2$, 1 mmol.L^{-1} DTT), pH 7.6. After that, genomic DNA from *N. caninum* (negative sample, 2 uL, 200 ng.µL^{-1}) or *T. gondii* (positive sample, 2 uL, 200 ng.µL^{-1}) were added and maintained at 52ºC for the duplex formation during 30 min.

3. Results and discussion

3.1. Isolation of DNA probe

The repetitive region described by Homan et al. [21] is a fragment of 529 bp, which is repeated 200 to 300 times in the genome of *T. gondii*. This fragment has been widely investigated as a molecular target for the diagnosis of toxoplasmosis [22-24].

The B1 gene is repeated 35 times in the parasite genome and has been commonly used for molecular diagnosis with acceptable sensitivity [24-27].

The dense granule antigens (GRA) are parasitic molecules secreted to the parasitophorous vacuole, being immunogenic and responsible for the parasite

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intracellular survival. GRA6 (dense granule antigens 6) protein is coded by GRA6 gene (a highly polymorphic single-copy gene) and used for genotyping [28-31]. Several studies have showed the use of recombinant antigenic proteins as GRA for serological diagnosis of toxoplasmosis [32-34].

In the probe selection process, Primer3 considered 5,397 possible probes for GRA6 gene, excluding 3,392 for undesirable secondary structure or long base repeats. The remaining 2,005 probes were sorted and the top scoring was submitted to BLAST alignment. For the repetitive region, from the 1,370 probes considered, 1,025 were excluded, resulting in 345 probes. For B1 gene, 6,429 probes were considered and 3,025 were excluded, resulting in 3,404 for scoring, sorting and alignment. Finally, the best result for each gene/genomic region was chosen. **Table 1** shows the sequences and highlights some of their features.

Among the probes selected, ToxG1 was chosen for immobilization on the modified graphite electrodes due to the higher percentage of guanine (G)-cytosine(C) content in relation to ToxR1 and ToxB1. A probe of higher GC content creates a strong bonder with complementary DNA.

Most tests used for the diagnosis of toxoplasmosis are based on the detection of different antibody classes or antigens. Other DNA-based technologies also use and rely on amplification procedures, based on Polymerase Chain Reaction (PCR) prior to detection. In this method, several multicopy and single-copy genes are used as targets [4]. Costa and collaborators [35] developed a nested-PCR assay using the GRA7 gene as target for prime design, which also encodes a dense granule protein. The test was sensitive and specific compared to other assays, as the methodology presented in this work.

3.2. Immobilization of the DNA probe

Conductive polymers have been widely used in the development of biosensors, due to various advantages, such as the presence of functional groups that facilitate the immobilization of biomolecules [36, 37]. A previous work carried out by our group shows that poly (3-HBA) is an efficient matrix for the oligonucleotide immobilization [12].

Graphite electrodes were modified with polymeric film derived from 3-HBA. A current peak was observed at +1 V, what is attributed to the monomer oxidation, which

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decreased after the potential scans. After the first cycle, a gradual increase in the current between +0.3 V and +0.9 V can be observed due to the coverage of the electrode with an electroactive material. This modification was confirmed by cyclic voltammetry, in which there is an increase in the current response after the electrode modification with poly (3-HBA).

After the production of the graphite electrode modified with poly(3-HBA), the sensitization with ToxG1 probe was carried out.

Surface density of probe is an important factor that determines the extent to which the immobilized probes are able to capture targets in solution [38-40]. The effect of probe concentration for immobilization (Fig. 2) was investigated, by the direct method, using the guanine residue oxidation signal.

Here Fig. 2

Figure 2A shows that the oxidation peak for guanine residue occurs at +0.96V *vs*. Ag/AgCl. With the increase in the guanine concentration, the current value of the oxidation peak for guanine increases until 25 μmol.L^{-1}, according to Figure 2B. This concentration was used in the hybridization experiments with the complementary target. These results show that ToxG1 probe was efficiently incorporated to the graphite electrode modified with poly(3-HBA).

3.3. Surface analysis using AFM

The probe immobilization (ToxG1) onto electrode surface was also confirmed using 3D AFM analysis (Fig. 3).

Here Fig. 3

Images of graphite electrode, graphite/poly(3-HBA) and graphite/poly(3-HBA)/ToxG1 probe present roughness values of 49.1 ± 2.0 nm, 27.4 ± 1.4 nm and 80.3 ± 3.6 nm, respectively. The surface of the electrode modified with polymer is smoother in comparison to the graphite electrode. Such figures indicate a greater uniformity of the modified electrode surface due to the fact that the polymer fills the valleys contained in the graphite electrode. After probe immobilization, both an increase in roughness and

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the appearance of clusters between 0.6 and 1.2 nm were observed according to the literature [41-42].

The effect of the probe concentration in the duplex formation is shown in Figure 4.

Here Fig. 4

A decrease in guanine oxidation peak current can be observed after hybridization with probe concentrations of 20 µmol.L^{-1} and 25 µmol.L^{-1}. However, as the difference between the probe (ToxG1) and the probe:target (ToxG1:ToxG2) was higher for 25 µmol.L^{-1}, this probe concentration was used in the experiments. In higher concentrations, it was observed to increase in the current response, probably due to the saturation of the surface by the probe, causing steric hindrance and making it difficult to interact with the complementary target.

The hybridization was monitored by the oxidation of guanine, which is the most redox active nitrogenous base in DNA strands. When hybridization occurs, there is a decrease in electrochemical signals due to the interaction of free guanines of the probe with complementary cytosine bases present in the target sequence [43]. Like the bioelectrode produced in this work, several label-free oligonucleotide biosensors have been described in literature [43-46], which are considered as the simplest method, once it does not require any special reagents or nucleic acid modifications.

3.4. Specificity studies

Specificity studies were conducted using non-complementary oligonucleotide and genomic DNA from *N. caninum*, human and *Mus musculus* as non-specific targets. ToxG2 or *T. gondii* were used as positive control (Fig. 5).

Here Fig. 5

When the non-specific targets (oligonucleotide or genomic DNA from *N. caninum*, human and *Mus musculus*) were added, the current values observed were similar to the probe. A decrease of about 20% in the guanine signal was observed due to

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the extent of hybridization between the ToxG1 probe in the presence of ToxG2 or *T. gondii* RH strain genomic DNA. This decrease is due to the formation of hydrogen bonds between the probe and the complementary target during the hybridization, which hampers the oxidation of nitrogenous bases [11, 16, 41- 43].

Figure 6 shows a decrease in current peak response with the increase in genomic DNA concentration and a linear relationship of the current peak with the genomic DNA concentration given by I/μA = -0.01817X + 19.81 (r = 0.9826), in the range of 100 ng.μL^{-1} to 400 ng.μL^{-1}. It was possible to detect up to 100 ng.μL^{-1} of the genomic DNA of *Toxoplasma gondii*.

Here Fig. 6

3.5. Analysis using AuNPs

Gold nanoparticles are a scaffold for DNA binding. The functionalization of AuNPs with oligonucleotides was used for the electrochemical analysis of the hybridization between ToxG1 probe: *T. gondii* DNA (positive sample) and ToxG1 probe: *N. caninum* DNA (negative sample) (Fig. 7).

Here Fig. 7

Figure 7a shows the absorption spectrum of the AuNPs. The peak of maximum absorbance at 520 nm is in accordance with Verissimo and collaborators [47].

When the ToxG1-S probe was incubated with the negative control (*N. caninum* DNA), both the absorbance peak and the displacement are similar to the probe (Fig. 7b), but with a lower intensity of ToxG1-S (Fig. 7c). However, when the ToxG1-S probe was incubated with the positive control (*T. gondii* DNA) (Fig. 7d), a significant change in the absorbance peak was observed, as well as widening and bathochromic shift, assuming a peak absorbance near 540 nm. The spectra in Figure 7d (shoulder in 580 nm) and Figure 7e (peak in 680 nm) suggest that the particles are close to each other, indicating an agglomeration process. These results indicate that the process of target recognition (genomic DNA) occurs by means of the biosensor, corroborating with the electrochemical results.

4. Conclusions

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An approach for the selection of DNA probes related to *T. gondii* was shown in this study. Poly(3-hydroxybenzoic acid) was effective for the immobilization and detection of DNA fragments of *T. gondii*.

The immobilization of the ToxG1 and the hybridization of the oligonucleotide (ToxG2) and genomic DNA showed good responses by direct detection of guanine residue oxidation. A decrease of about 20% in the guanine signal was observed due to the hybridization between the ToxG1 probe in the presence of ToxG2 or *T. gondii* genomic DNA. In the presence of non-specific targets (*N. caninum*, human and *Mus musculus*), the current values observed were similar to the probe.

Hybridization was also confirmed by optical assays using AuNPs. When the ToxG1S probe is incubated with the *T. gondii* genomic DNA, there is a significant change in the absorbance peak, widening and shifting to a longer wavelength in accordance with the electrochemical results.

The results obtained contribute to the further development of the rapid diagnosis of *T. gondii* infection or toxoplasmosis.

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Figure captions

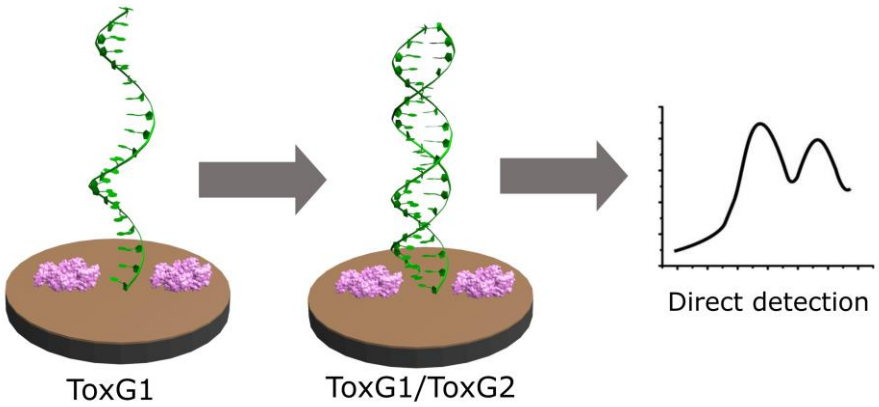

Figure 1. Schematic representation for the immobilization of the ToxG1 probe on modified electrode and its hybridization.

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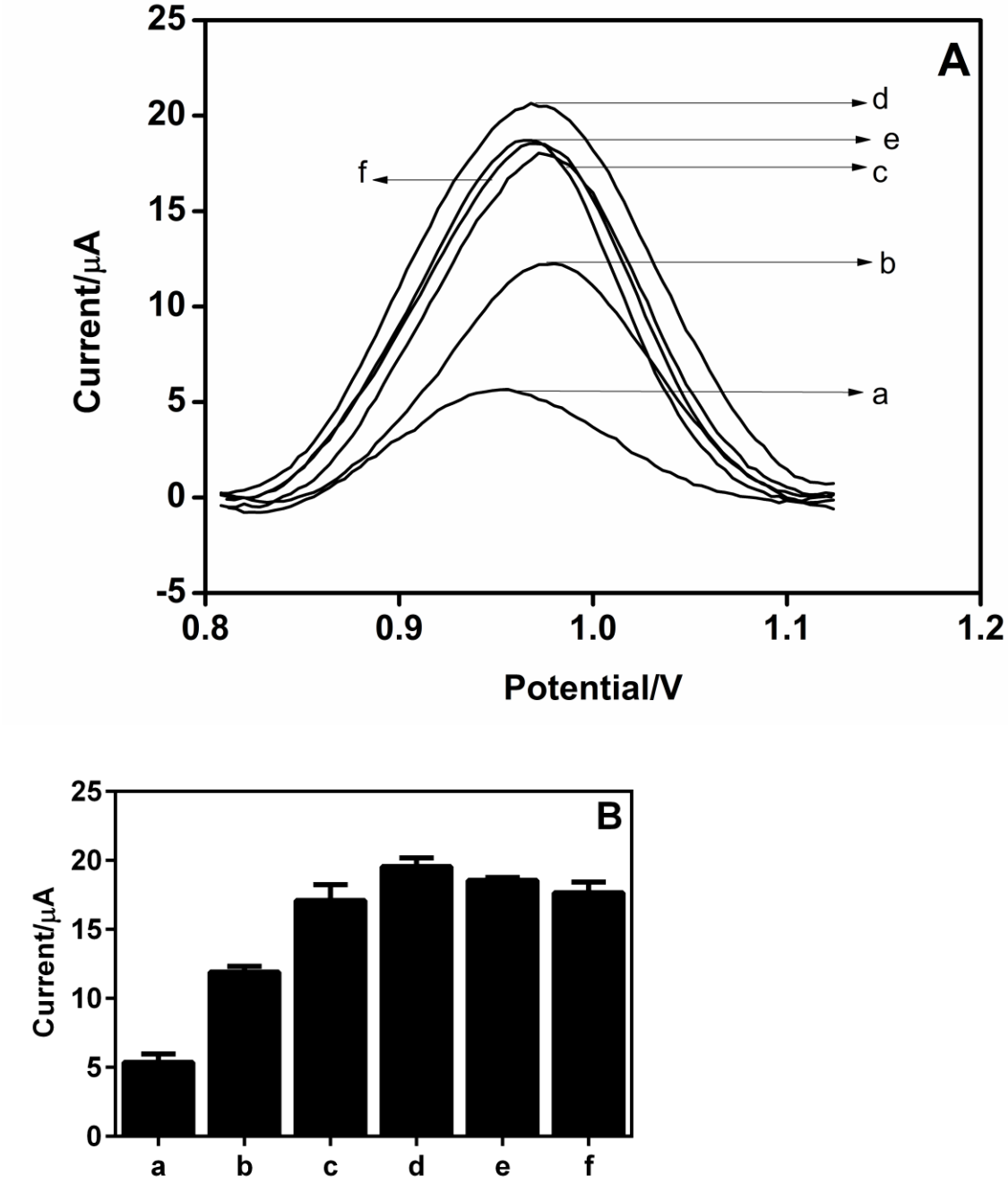

Figure 2. Differential pulse voltammograms (A) and histograms of the peak current response (B) of guanine residue oxidation in graphite electrodes modified with poly (3-HBA) after immobilization of different concentrations of probe (ToxG1): 5 μmol.L^{-1} (a) 15 μmol.L^{-1} (b) 20 μmol.L^{-1} (c) 25 μmol.L^{-1} (d) 35 μmol.L^{-1} (e) and 50 μmol.L^{-1} (f). Modulation amplitude: 25 mV, pulse interval: 0.2 s, scan rate: 20 mV.s^{-1}.

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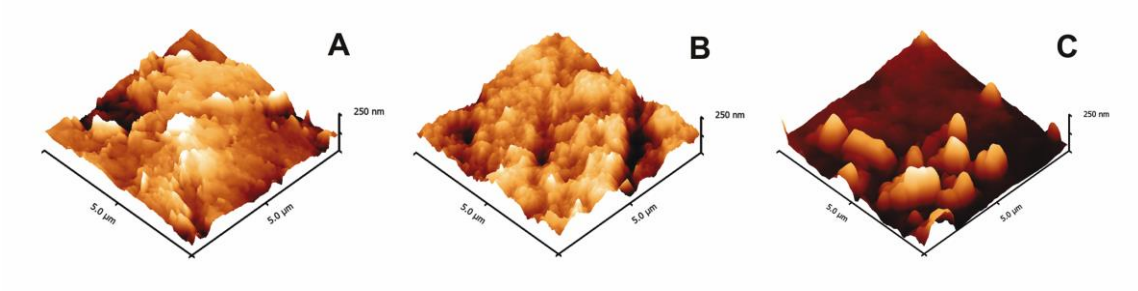

Figure 3. AFM topographical images of graphite electrode (A); graphite/poly (3-HBA) (B); graphite/poly (3-HBA)/probe (C).

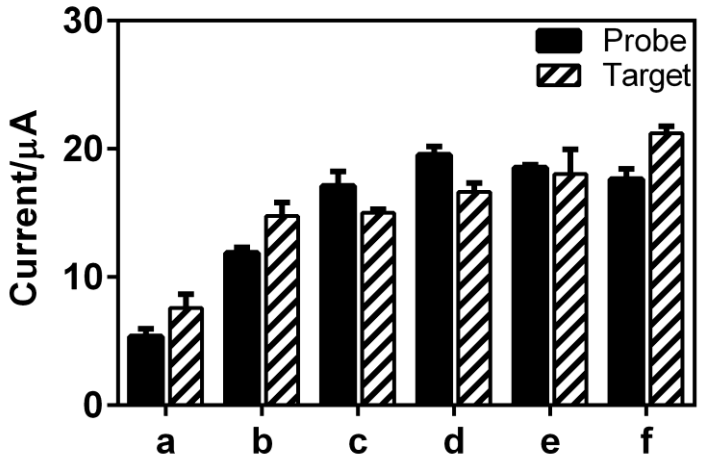

Figure 4. Histogram obtained from current peak response of guanine oxidation in graphite electrodes modified with poly (3-HBA) after immobilization of different concentrations of probe (ToxG1): 5 µmol.L^{-1} (a) 15 µmol.L^{-1} (b) 20 µmol.L^{-1} (c) 25 µmol.L^{-1} (d) 35 µmol.L^{-1} (e), 50 µmol.L^{-1} (f) and after 15 min of incubation with different concentrations of complementary target (ToxG2): 10 µmol.L^{-1} (a) 30 µmol.L^{-1} (b) 40 µmol.L^{-1} (c) 50 µmol.L^{-1} (d) 70 µmol.L^{-1} (e) and 100 µmol.L^{-1} (f).

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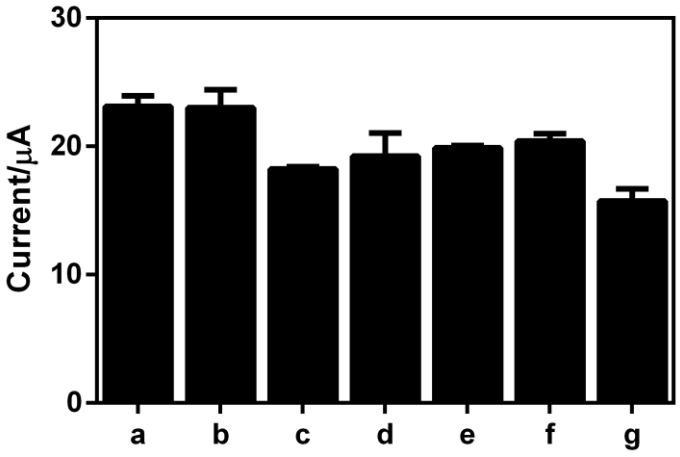

Figure 5. Bar chart obtained from differential pulse voltammograms of guanine residue onto graphite electrode modified with poly (3-HBA) containing ToxG1 probe, before hybridization (a) and after hybridization with: non-complementary oligonucleotide (b), complementary target oligonucleotide (ToxG2) (c), *Neospora caninum* genomic DNA (d), human genomic DNA (e), *Mus musculus* genomic DNA (f) and *Toxoplasma gondii* genomic DNA (g). Electrolyte: phosphate buffer (0.10 $mol.L^{-1}$), pH 7.4, modulation amplitude: 25 mV. Pulse interval: 0.2 s; Scan rate: 20 $mV.s^{-1}$.

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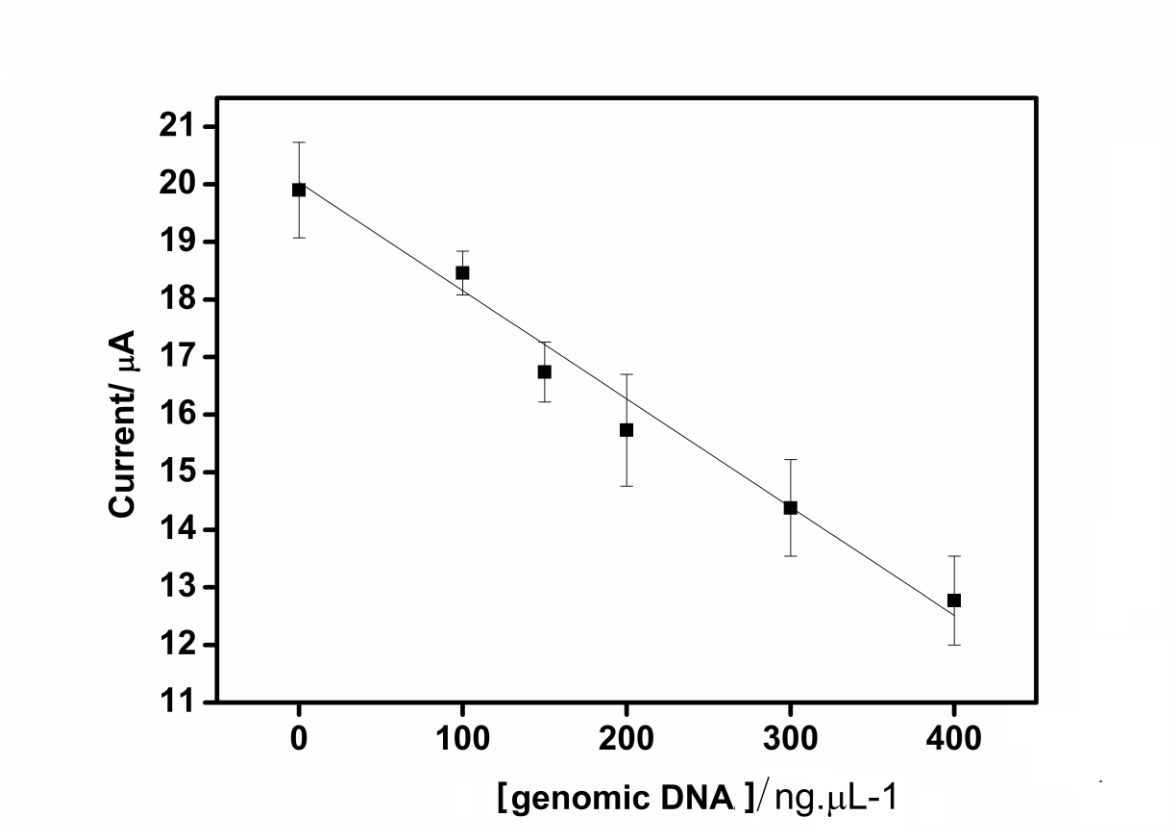

Figure 6. Calibration curve for the oxidation signal of guanine residue obtained from the differential pulse voltammograms onto graphite electrode modified with poly(3-HBA), containing ssDNA (TOXG1) before and after hybridization with different concentrations of *Toxoplasma gondii* RH strain genomic DNA (100 ng.μL^{-1} to 400 ng.μL^{-1}). Electrolyte: phosphate buffer (0.10 mol.L^{-1}), pH 7.4. Modulation amplitude: 25 mV. Pulse interval: 0.2s; 20mVs^{-1}.

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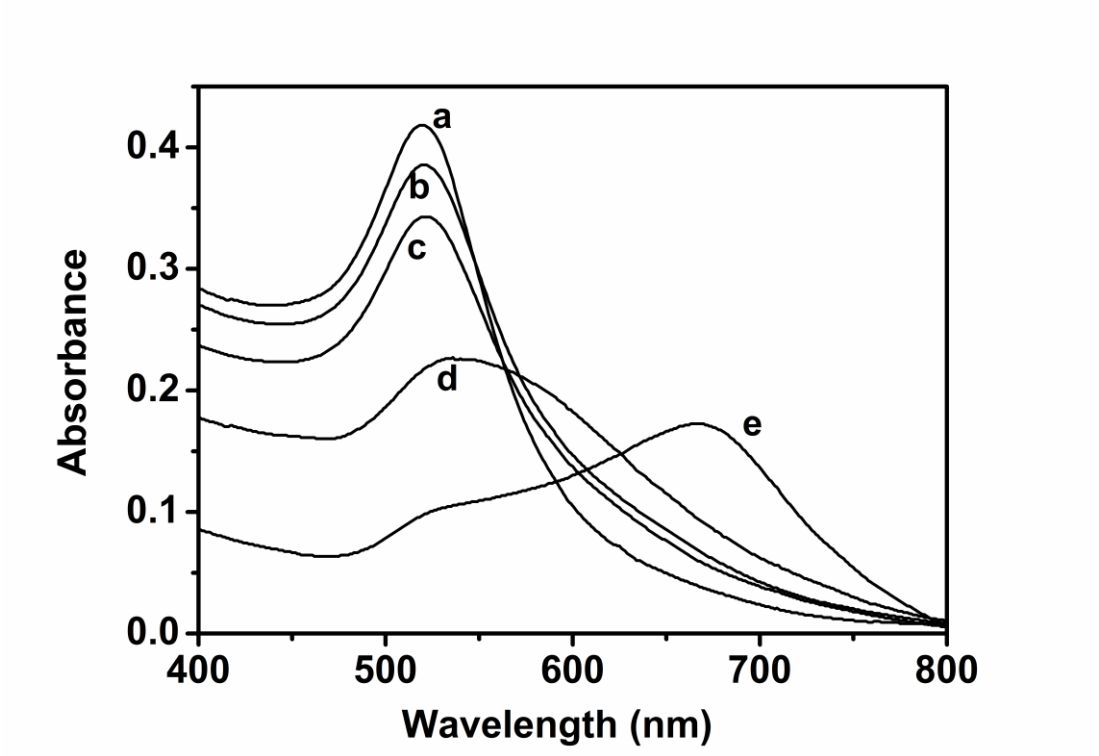

Figure 7. Absorption spectrum in the UV-vis of: AuNPs (a), AuNPs/TOXG1/NaCl (saturated) (b), AuNPs/ToxG1/ *Neospora caninum*/NaCl (saturated) (c) AuNPs/ToxG1/ *Toxoplasma gondii*/ NaCl (satured) (d) and AuNPs/NaCl (saturated) (e).

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Table 1. Selected probes and their features.

		Primer3 features						Blast best hit features		
ID	Genomic target	LEN	GC%	TM	SELF-ANY	SELF-END	HAIRPIN	Query cover	Identity	E-value
ToxG1	GRA6 gene (L33814.1)	21	57,14	69,3	0	0	0	100%	100%	0.051
ToxR1	Repetitive region (AF146527.1)	22	54,55	68,83	0	0	0	100%	100%	0.019
ToxB1	B1 gene (AF179871.1)	21	42.62	66.62	0.81	0	0	100%	100%	0.051

* LEN: oligonucleotide length, GC%: GC content, TM: melting temperature, self-any: tendency of any self-complementarity, self-end: tendency of ends self-complementarity, hairpin: tendency of hairpin formation, ToxG1 oligonucleotide sequence: 5'-CTGGGAACGGTGGGAATGAAG-3', ToxR1 oligonucleotide sequence: 5'-GGCGGAGAGAATTGAAGAGTGG-3', ToxB1 oligonucleotide sequence: 5'-AAATGCCAGAAGAAGGGTACG-3'