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Magnetic bead/gold nanoparticle double-labeled primers for electrochemical detection of isothermal amplified Leishmania DNA

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ABSTRACT

A novel methodology for the isothermal amplification of *Leishmnania* DNA using labeled primers combined with the advantages of magnetic purification/preconcentration and the use of gold nanoparticle (AuNPs) tags for the sensitive electrochemical detection of such amplified DNA is developed. Primers labeled with AuNPs and magnetic beads (MBs) were used for the first time for the isothermal amplification reaction, being the amplified product ready for the electrochemical detection. The electrocatalytic activity of the AuNP tags

towards the hydrogen evolution reaction allowed the rapid quantification of the DNA on screen-printed carbon electrodes.

Amplified products from blood of dogs with *Leishmania* (positive samples) were discriminated from those of healthy dogs (blank samples). Quantitative studies demonstrate that the optimized method allows to detect less than one parasite per microliter of blood (8 x 10^{-3} parasites in the isothermal amplification reaction). This pioneer approach is much more sensitive than the traditional methods based on real-time PCR, being also more rapid, cheap and user-friendly.

1. Introduction

Diseases transmitted by blood-feeding vectors (parasites) are a growing threat to world human health, particularly those affecting pets and transmitted by fleas, ticks, sandflies or mosquitoes (Vector Borne Diseases, VBD).[1,2,3] One of the most important VBD is visceral leishmaniasis which is endemic in 88 countries on 4 continents.[4,5,6] Zoonotic visceral leishmaniasis, caused by the protozoan *Leishmania infantum* and transmitted by sandfly vectors, is a fatal disease of domestic dogs, wild canids and humans. [7] Different methods for the detection and diagnosis of Canine Leishmaniasis (CanL) including parasitological,[8-10] serological[11,12] and molecular techniques[13-19] have been reported, suffering of limitations related to the need of skilled workers, the high cost and the fact that samples must be send to a reference lab. Lateral-flow assay (LFA) strips for specific CanL antigen are commercially available for detection of visceral leishmaniasis.[20-23] However, the presence of low antibody levels is not necessarily indicative of disease and further work-up is necessary to confirm it by DNA-based diagnostic methods, being PCR the gold standard. In this context, a dipstick format was developed for the detection of PCR amplified CanL DNA.[24] A qualitative real-time PCR (Leish PCR

assay)[25] is also commercially available for diagnosing cutaneous leishmaniasis. However, both systems did not overcome the limitations of PCR for which sophisticated and expensive equipment is needed to perform the precise and repeated heating cycles required. Therefore, there is still a need for further point-of-care (POC) diagnostic methods for the detection of infections by pathogens, particularly for leishmaniasis.

Isothermal amplification is an alternative approach to the traditional PCR which overcomes many of the complications related to the thermocycling since it is performed at a constant temperature thanks to the use of enzymes. There are many different isothermal amplification methods, depending on the enzymes and the temperature used. The variation called Recombinase Polymerase Amplification (RPA) commercialized by Twist (TwistDx's®)[26] employs recombinase enzymes which are capable of pairing oligonucleotide primers with homologous sequence in duplex DNA typically within 5 - 10 minutes. However, in most cases DNA purification and detection after amplification still requires hazardous, time consuming and expensive equipment, giving only qualitative information, so alternative methodologies overcoming such problems are required. In this context, the integration of nanoparticle (NP) tags during the amplification process combined with the very sensitive electrocatalytic[27,28] detection of such a tag would overcome most of these limitations. The outstanding properties of different nanomaterials have been extensively approached in DNA biosensing systems. NPs have been used in a high extent as both optical and electrochemical tags in DNA hybridization biosensors[29-37] while magnetic beads (MBs) have been extensively used as platforms of such and other bioassays.[38,39] Gold nanoparticles (AuNPs)[40,41] and in a minor extent silver nanoparticles (AgNPs)[42] and quantum dots (QDs)[43] have been also introduced in the cocktail of reagents of the PCR, taking advantage of their properties as catalyzers of the DNA amplification reaction, constituting the so-called nanoPCR[44] in some

cases after MB-based DNA extraction.[45] Only a report on AuNPs-labeled primers for optical detection of PCR amplified DNA is found in the bibliography[46] while few examples of PCR amplification using MB-labeled primers[47-49] have been published, but the integration of both MBs and AuNPs-labeled primers have not ever been reported, probably due to the high temperature reached during the PCR cycle which is a serious limitation for preserving the labeled primer integrity. Regarding the isothermal DNA amplification, AuNPs have been used as reporters in optical approaches based on AuNPs addition after DNA amplification followed by colorimetric[50-55] or surface plasmon resonance-based[56,57] detection, in some cases taking also advantage of MBs after the amplification reaction for pre-concentration/purification purposes.[58] In a similar way, AuNPs have been used as electrochemical reporters in DNA hybridization biosensors for the detection of isothermal amplified DNA[59] taking also advantage of the use of MBs platforms.[60,61] All these approaches are based on the addition of the micro/nanoparticles after the DNA amplification and in most cases require the performance of further DNA hybridization assays, which increase the analysis time and also involve more irreproducibility, loss of sensitivity and false positives due to unspecific absorptions.

In this work, we present a novel design of *Leishmania* DNA isothermal amplification using for the first time primers labeled with both AuNPs and MBs. The low and constant temperature of the isothermal amplification procedure is ideal for preserving the integrity of the nanoparticle-primer conjugates during the amplification step. The double-labeled product resulting from such amplification is ready for a rapid magnetic separation/pre-concentration and direct electrocatalytic detection in a very sensitive and quantitative way. A general scheme of the whole experimental procedure from the DNA extraction to the final electrochemical detection is shown in **figure 1**.

2. Results and discussion

Design and screening of primer sets for Leishmania assay and endogenous control

3 candidate primers for kinetoplast and 8 candidate primers for Intergenic Spacer region (ITS1) of *Leishmania* were designed following recommended conditions (Appendix to the TwistAmpTM reaction kit manuals[26]). All possible combinations of primers were tested using standard conditions of TwistAmp (37ºC of reaction temperature; Magnesium-Acetate concentrations of 14 mM; shaking 4' after initiation of the reaction; 480 nM of each primer). Parameters that can be modified by the user (temperature, magnesium concentration, stirring regime and primer concentrations) were adjusted to obtain the best performance. This first step generates 4 sets of primers used in simplified standard conditions (37ºC of reaction temperature; at 14 mM of Magnesium concentration, 480 nM of primer concentration, without agitation 4' after starting the reaction and with a reaction time of 20'). To improve the performance of the *Leishmania* assay a 'second generation' of primers was designed by creating variants of the best primer set identified in the first step (moving 1 base pare around the initial primer) and re-screening the new candidates to improve amplification performance. Finally, with definitive ones, primer concentration was adjusted to 300 nM and reaction time was decreased up to 10' (**figure 2a** and **b**).

An endogenous control was incorporated to avoid the generation of artifacts that were detected in no-template controls and samples of very low target copy number. To optimize the endogenous control RPA amplification 6 candidate primers for 18S ribosomal RNA gene were screened (**figure 2c**).

The assay presented based on isothermal co-amplification of *Leishmania* parasite and 18S as an endogenous control exhibits similar results than the real time used as a gold standard,[62] with a limit of detection of <1 parasite in the reaction. It is possible since both assays target a

conserved region of the *Leishmania* kinetoplast minicircle DNA that is present in about 10.000 copies for parasite.[63-65] Multiplex isothermal has been optimized with an endogenous control primer concentration limited to 150 nM to avoid a loss of sensitivity when low targets of *Leishmania* were present.

Characterization of AuNP/amplified DNA

The performance of the isothermal amplification procedure using primers labeled with AuNPs was evaluated by both Zeta potential and electrochemical measurements. Zeta potential is well known as an efficient tool for the monitoring and analysis of modifications on the surface of NPs, with minimal sample preparation.[66] It has been used to obtain information concerning the particle surface charge, chemical modifications and also stability of colloid suspensions. A high zeta potential (positive or negative; typically higher than 10 mV) confers stability since the dispersion resists aggregation. Since ssDNA is negatively charged, the conjugation of AuNPs with ssDNA should give rise to negative charged conjugates which would shift the Z potential to more negative values. All these characteristics make zeta potential an ideal technique for the characterization of both the primers and the final amplified DNA labeled with AuNPs.

As can be observed in **figure 3a**, a gradual shift to more negative values was observed in the Zeta potential of AuNPs when higher was the coverage degree by ssDNA. This suggests that AuNPs were being loaded with negative charged molecules such as ssDNA. First, a shift from the value of the non-modified AuNPs (a) was observed for the AuNP/primer (b) indicating that the primer was correctly connected with the AuNP. After the amplification, a shift of up to 25 mV was observed (d), suggesting the covering of the AuNPs with the amplified dsDNA

(that has more negative charges). No significant shift was observed for a control amplification assay performed with a blank sample (c), also corroborating the specificity of the system.

These evidences were also corroborated by electrochemical measurements. Three different "positive" and "blank" samples were analyzed for this purpose. The AuNP/amplified DNA product was purified by incubation with streptavidin-modified MBs which capture the amplified products through the biotin present in the primer and finally detected taking advantage of the electrocatalytic activity of AuNPs toward the hydrogen evolution reaction (HER). Briefly, this well-known methodology is based on the fact that the presence of AuNP connected to MBs on the SPCE surface shifts the potential for hydrogen ion reduction toward less negative potentials. Fixing a reductive potential of -1.00 V, the intensity of the current recorded in chronoamperometric mode during the stage of hydrogen ion electroreduction at 60 seconds (chosen as analytical signal) is related to the quantity of AuNPs on the SPCE and consequently to the amount of tagged analyte.[27,28]

As observed in **figure 3b**, "positive" and "blank" samples can be perfectly discriminated in the three samples, suggesting the specific presence of AuNPs in the amplified products and consequently the good performance of the isothermal amplification in the presence of such tags. The signals of the "blank" samples are at the normal levels of background of the HER in absence of catalyzer (around 5-7 μA), so the specificity of the amplification is demonstrated.

Furthermore, it was observed that the life-time of AuNP/primer conjugates was of up to 8 weeks when stored at 4ºC and protected from light, as shown in **figure 3c** where different amplifications for "positive" samples were performed at different times after the preparation of the AuNP/primer conjugate. The dramatic decrease in the analytical signal observed after 8 weeks suggests the damaging of the AuNP/primer, probably due to either breaking of the

binding or AuNPs agglomeration. Further studies will be focused on alternative AuNP/primer storage conditions, such as freezing or lyophilization.

Evaluation of double labeled MB-AuNP/amplified DNA

Once demonstrated the good performance of the isothermal amplification procedure using AuNP-labeled primers, the experimental procedure was significantly simplified by also introducing the MB-labeled primer in the amplification reagents mixture as illustrated in **figure 1**. In this case, the experimental procedure for the measurement after the amplification is enormously simplified since the MB/amplified DNA/AuNP complex is directly placed onto the SPCE surface and captured by the magnet placed on the reverse side of the working electrode immediately before the electrochemical measurement.

MBs of two different sizes (2.8 µm and 1 µm) and two different kind of primers were evaluated: the K3R detailed in the experimental section and also a longer one that includes a spacer of $(AT)_7$ in the 5' tail. This tail of oligonucleotides helps to keep the magnetic bead far from the amplification zone.

The results shown in **figure 4a** seem to indicate that the amplification doesn't work properly using the bigger MBs in combination with the short primer since the recorded currents are in the levels of the background. The results improved a little bit when the use of longer primers (containing a spacer oligonucleotides sequence) was introduced. When the smaller MBs and the short primer were tested, the amplification worked in a higher extent, and this was highly improved when it was used in combination with the primers containing a spacer. This suggests that the bigger MBs are hindering the amplification, probably due to the beads deposition during the amplification procedure. Furthermore, the procedure takes advantage in

this case of the fact that the amplification sequence of the primer is not in direct contact with the bead, which probably facilitates the DNA amplification.

The good performance of the amplification procedure as well as the integrity of the double-labeled amplified products was evaluated by SEM analysis. As shown in **figure 4b**, AuNPs (observed as small white spots) are connected to the MBs (big microspheres in the image) after the amplification procedure, evidencing the formation of the MB-AuNP/amplified DNA (a control image after amplification of DNA without *Leishmania* parasite is shown at the Supporting Information).

Quantitative assays: evaluation of products with different parasite concentration

The optimized methodology was applied for the evaluation of amplified DNA prepared from samples containing different quantities of spiked parasite. Chronoamperograms recorded for the assayed samples are shown in the inset of **Figure 5a**, where the values of the analytical signals (current at 60 seconds) after subtracting the background (current in 1M HCl, in the absence of catalyzer) are represented for a better understanding.

As shown in **Figure 5b**, a linear relationship between the analytical signal (after subtracting the background current) and the logarithm of parasite concentration in the range 500 to 0.5 parasites per mL of blood (samples prepared as detailed in the experimental section) was obtained. A limit of detection (LOD) of 0.8 parasites per mL of blood (8×10^{-3} parasites per DNA amplification reaction) was estimated, as the parasite number giving a signal equal to the blank signal plus three times its standard deviation. The reproducibility of responses for 5 parasites per mL of blood was also studied, obtaining a relative standard deviation (RSD) of 7%.

Our AuNP/MBs based electrochemical approach results are quite better to those obtained by other POC tests using nucleic acid sequence based amplification (NASBA) and coupled to oligochromatography (OC) for *Leishmania* detection[67] and even much better than the 1 parasite per PCR detection limit offered by the OligoC-test®.[24]

3. Conclusions

In summary, a novel design of isothermal amplification using for the first time primers labeled with both AuNPs and MBs for obtaining of double labeled amplified products has been successfully developed for a DNA sequence characteristic of *Leishmania infantum* kinetoplast tested in dogs, chosen as model. The double label allows the rapid magnetic purification/preconcentration of the product followed by direct AuNP electrocatalytic detection, avoiding DNA hybridization procedures. The electrochemical method exhibits a good reproducibility and sensitivity, allowing to detect 0.8 parasites per mL of blood (8×10^{-3} parasites per DNA amplification reaction). Furthermore, amplified DNA from dogs without *Leishmania* can be perfectly discriminated, demonstrating the specificity of both the amplification procedure and the electrochemical detection. In addition to the advantages of simplicity and one-step detection of the amplified product, the performance of the proposed approach is better than the obtained with other point-of-care tests for Leishmania detection, offering also a quantitative tool for parasite determination.

Furthermore, our technique provides a valuable proof of concept since the double MB/AuNP-label approach is a universal methodology that could be applied for any RPA isothermal DNA amplification design.

4. Experimental section

DNA samples

The present study includes samples of dogs received in Vetgenomics (www.vetgenomics.com) to perform *Leishmania* detection by real time PCR. DNA was isolated from 400 µL of EDTA- blood samples using the Genelute blood Genomic DNA Kit (Sigma, Spain) following manufacturer instructions. DNA was re-suspended in 200 µL of elution buffer. A *Leishmania* positive DNA was used to perform the different isothermal amplifications and a negative DNA (without *Leishmania*) was used as blank.

To perform the calibration curve a negative blood (400 µL) was spiked with 2 µL of *Leishmania* culture at different concentrations, ranging from 10^5 to 10^2 promastigotes/mL. Samples assayed consequently range from 500 to 0.5 parasites per mL of blood (from 5 to 5 x 10^{-3} parasites per isothermal amplification reaction in a tenfold dilution).

Chemicals and equipment

Primers labeled with biotin and thiol were purchased from Sigma (Spain). TwistAmp® Basic Kit containing all enzymes and reagents necessary for the amplification of DNA – was supplied by TwistDx Ltd (UK). 16 nm-sized AuNPs were prepared following the Turkevich's method (see detailed preparation procedure, TEM characterization and electrocatalytic quantification of AuNPs at the Supporting Information). High monodisperse 1 µm and 2.8 µm sized streptavidin-coated magnetic beads (MBs) were purchased from Dynal Biotech (MyOne and M-280 respectively, Invitrogen, Spain) (see SEM characterization of both beads at the Supporting Information). Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \bullet 3H_2O$, 99.9%) and trisodium citrate ($Na_3C_6H_5O_7$), used in the synthesis of AuNPs and tris (2-carboxyethyl) phosphine (TCEP) used for thiolated primer pretreatment were purchased from

Sigma-Aldrich (Spain). Washing solutions of the MB-labeled conjugates consisted of: (i) Phosphate buffer solution (PBS buffer): 0.01 M phosphate-buffered saline, 0.137 M NaCl and 0.03 M KCl (pH 7.4), (ii) Binding and washing buffer (B&W): PBS buffer solution with added 0.05% (w/v) Tween 20; (iii) Blocking buffer solution: 5 % (v/v) bovine serum albumin (BSA) in PBS buffer. A TS-100 ThermoShaker (Biosan, Latvia) was used for the incubations with agitation at a controlled temperature. A Dynal MPC™-S Magnetic Particle Concentrator (Invitrogen, Spain) was used for the magnetic separations.

The electrochemical transducers used were homemade screen-printed carbon electrodes (SPCEs), consisting of three electrodes: carbon working electrode (WE), Ag/AgCl reference electrode (RE) and carbon counter electrode (CE) in a single strip fabricated with a semi-automatic screen-printing machine DEK248 (DEK International, Switzerland) (see Supporting Information). The materials and reagents used for this process were: Autostat HT5 polyester sheet (McDermid Autotype, UK), Electrodag 423SS carbon ink, Electrodag 6037SS silver/silver chloride ink and Minico 7000 Blue insulating ink (Acheson Industries, The Netherlands). The experimental procedure for SPCEs fabrication is detailed at the Supporting Information. A neodymium magnet (3 mm in diameter), inserted under the WE, was also used to accumulate the MB-labeled amplified product during the electrochemical measurements. The electrochemical measurements were performed in analytical grade 1 M HCl solution (Merck, Spain) at room temperature using a µAutolab II (Echo Chemie, The Netherlands) potentiostat/galvanostat connected to a PC and controlled by Autolab GPES 4.9007 software (General Purpose Electrochemical System). Unless otherwise stated, all reagents and other inorganic chemicals were supplied by Sigma-Aldrich or Fluka (Spain).

All chemicals were used as received and all aqueous solutions were prepared in milli-Q water (Millipore purification system, 18.2 MOhm cm).

Conjugation of AuNPs and magnetic beads (MBs) to DNA primers

First of all, a reduction step to remove the protecting disulfide group from the thiolated primer was performed before using, following the protocol recommended by the manufacturer. Briefly, 200 μL of 10 mM Tris (2-carboxyethyl) phosphine (TCEP) were added to the lyophilized primer and the solution was shaked 60 min at room temperature. Then it was precipitated by adding 150 μL of 3M NaAc and 750 μL of EtOH and incubating 20 min at -20ºC. After that, it was spinned for 5 min at 13000 rpm and the supernatant was discarded. The pellet was dried at room temperature.

The conjugation of self-synthesized 16 nm-sized AuNPs to the primer modified with thiol group was performed adapting the procedure pioneered by Mirkin et al.[68] 190 μL of AuNPs suspension were mixed with 10 μL of 1500 μg/mL thiolated primer solution and incubated for 20h at 25 ºC with agitation (250 rpm) (final concentration of primer: 75 μg/mL). After that, this solution was added to 50 μL of 10 mM phosphate buffer (pH 7) / 0.1M NaCl and allowed to stand for 44h. Finally, a centrifugation at 14000 rpm for 20 min at 4ºC was carried out, and AuNPs/ primer conjugate were reconstituted in 200 μL of milli-Q water, being stable for up to 8 weeks.

Steptavidin-modified MBs (1 μm and 2.8 μm-sized) were connected with the primers through the streptavidin-biotin interaction by mixing 50 μL of 1 mg/mL MBs suspension with 15 μL of 100 μM biotinylated primer during 30 min at 25ºC. The MBs/ primer conjugate was washed and re-suspended in 15 μL of water.

Primer sets for Leishmania amplification and endogenous control

Primers set selected after optimization were (5'-3'):

- K6F: [ThiC6]CTTTTCTGGTCCTCCGGGTAGGGGCGTTCTG
- K3R: [Btn]CCACCCGGCCCTATTTTACACCAACCCCCAGTTTCCC

that amplified a fragment of approximately 140 base pair length of the Kinetoplast.

For the endogenous control, optimum primers were (5'-3'):

- Primer 18s 1F: CTGCGAATGGCTCATTAAATCAGTTATGGTTCC
- Primer 18s 1R: CTGACCGGGTTGGTTTTGATCTGATAAATGCACGC

that amplified a fragment of 168 base pair length of the 18S ribosomal RNA gene.

Analysis of RPA amplification was performed on 2% agarose-gel stained with ethidium bromide after purification of the reactions with the DNA Clean & Concentrator Kit (Zymo research).

Isothermal amplification assay conditions

Multiplex amplification with primers K6-F and K3R for *Leishmania* and 18S-F1 and 18S-R1 for endogenous control was performed using 14mM of magnesium, 300 nM of *Leishmania* primers, 150nM of 18s primers, 5µL of DNA and 37ºC of reaction temperature during 10 minutes without agitation.

The same experimental procedure was followed for K6F/AuNPs and K3R/MBs labeled primers (prepared as stated before) so as to obtain the double-labeled amplified product. A preliminary evaluation of the amplification using K6F/AuNPs and free K3R (only modified with biotin) was also performed. In this case, the AuNP/amplified DNA was purified by incubation with MBs under the same conditions than the described for the conjugation of MBs with biotin-labeled primers.

A sample without DNA was used in each isothermal amplification as a Negative Template Control (NTC).

Zeta potential measurements

A 1 μL suspension of AuNPs, K6F/AuNPs and of AuNPs/amplified DNA was diluted in 1 mL of PBS buffer, vortexed, and transferred into a 4 mL polystyrene cuvette (FB55143, Fisher Scientific). The data were collected and analysed with the Dispersion Technology software 4.20 (Malvern) producing diagrams for the zeta potential as a distribution versus total counts.

Electrochemical detection of MB/AuNP-labeled amplified DNA

25 μL of the MBs/amplified DNA/AuNPs complex suspension were placed on the working area of the SPCE, where it was previously attached a magnet on the reverse side. After 30 seconds, 25 μL of 2M HCl solution were added and a potential of +1.35 V was applied during 1 min (electrochemical pre-treatment). After that, a potential of -1.00 V was applied during 60 seconds in chronoamperometric mode. Under these conditions, the H^+ ions were reduced to H_2 thanks to the catalytic effect of the AuNPs labels.[27,28] The absolute value of the current registered at 60 seconds was considered as the analytical signal, being this value proportional to the quantity of AuNPs and, consequently, to the concentration of isothermal amplified product. For the quantitative study shown in **figure 5**, the values of the analytical signals (current at 60 seconds) after subtracting the background (current in 1M HCl) are plotted for a better understanding.

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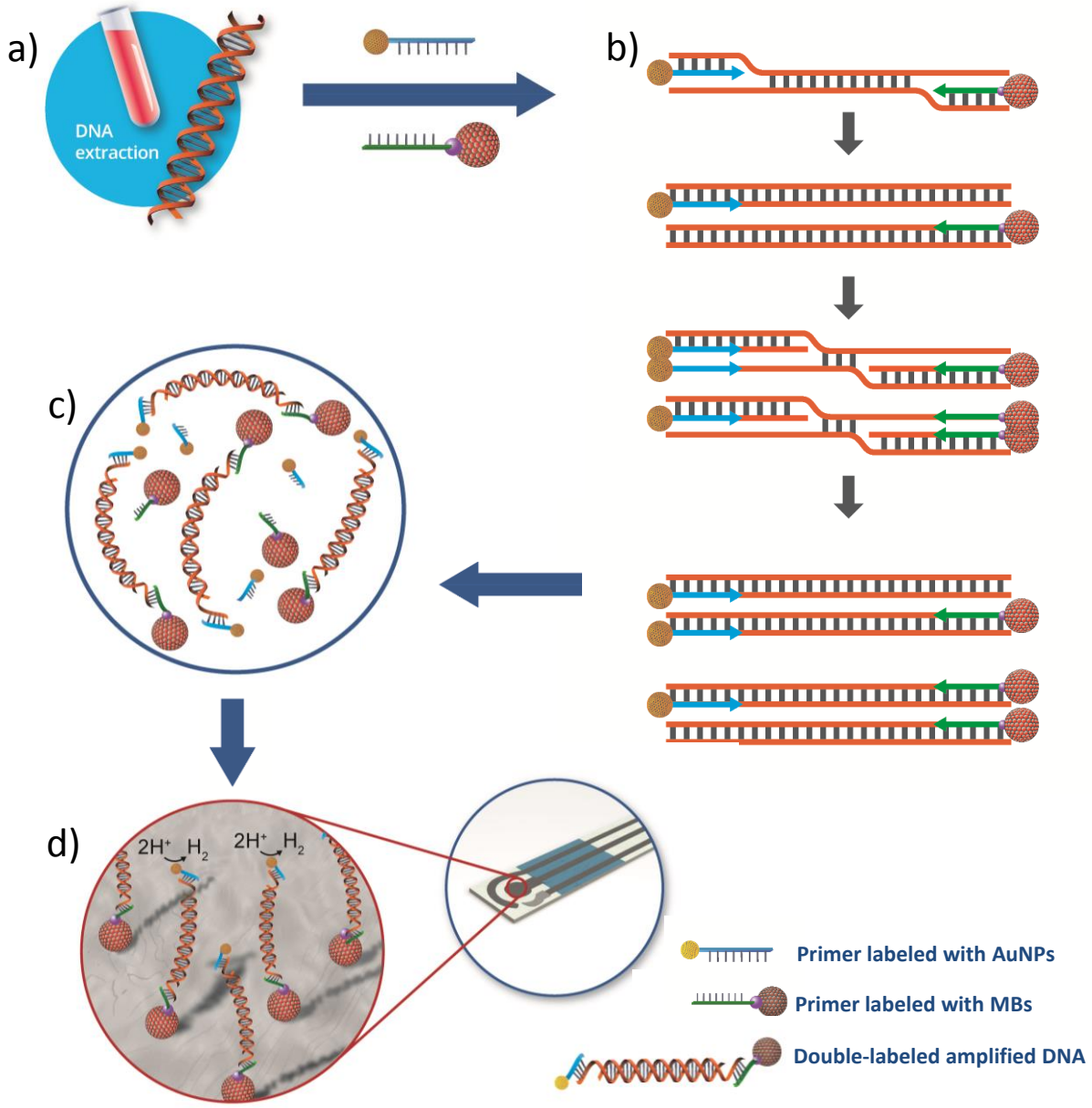

Figure 1: Scheme of the experimental procedure for the detection of isothermal amplified DNA using primers labeled with AuNPs and MBs. DNA is extracted from dog blood **(a)** and a kinetoplast specific region is isothermal amplified by RPA cycle using primers labeled with AuNPs and MBs **(b)**. The double-labeled amplified product (MB/amplified DNA/AuNP complex) **(c)** is captured by the magnet placed on the reverse side of the working electrode of the SPCE and AuNPs tags are detected through the electrocatalytic Hydrogen Evolution Reaction (HER) **(d)**.

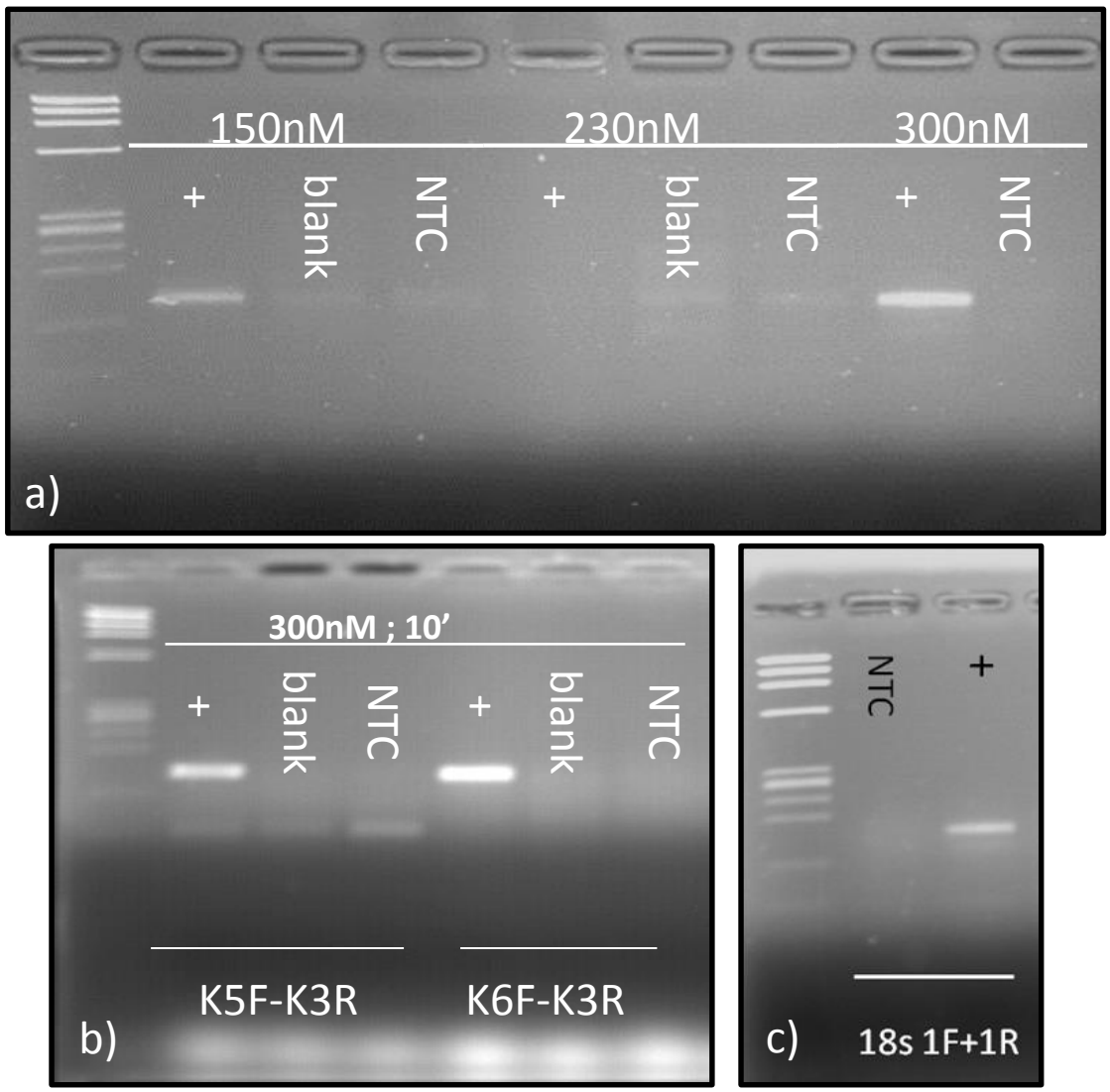

Figure 2.Isothermal assay optimization evaluation by gel electrophoresis. **(a)** Optimization of primer concentration of *Leishmania* assay.; **(b)** Example of the "second generation" screening of *Leishmania* primers: K6F-K3R are the chosen ones for its best performance; **(c**) Definitive endogenous control assay used. +, positive sample (DNA of a dog with *Leishmania* infection); blank, (DNA of a dog without *Leishmania* infection); NTC, negative template control. Line 1 of each gel: Phi-X 174/HaeIII Marker (1353/1078/872/603/310/(281,271)/234/194/118/72). Bands size: 140 bp for *Leishmania* and 168 for 18S.

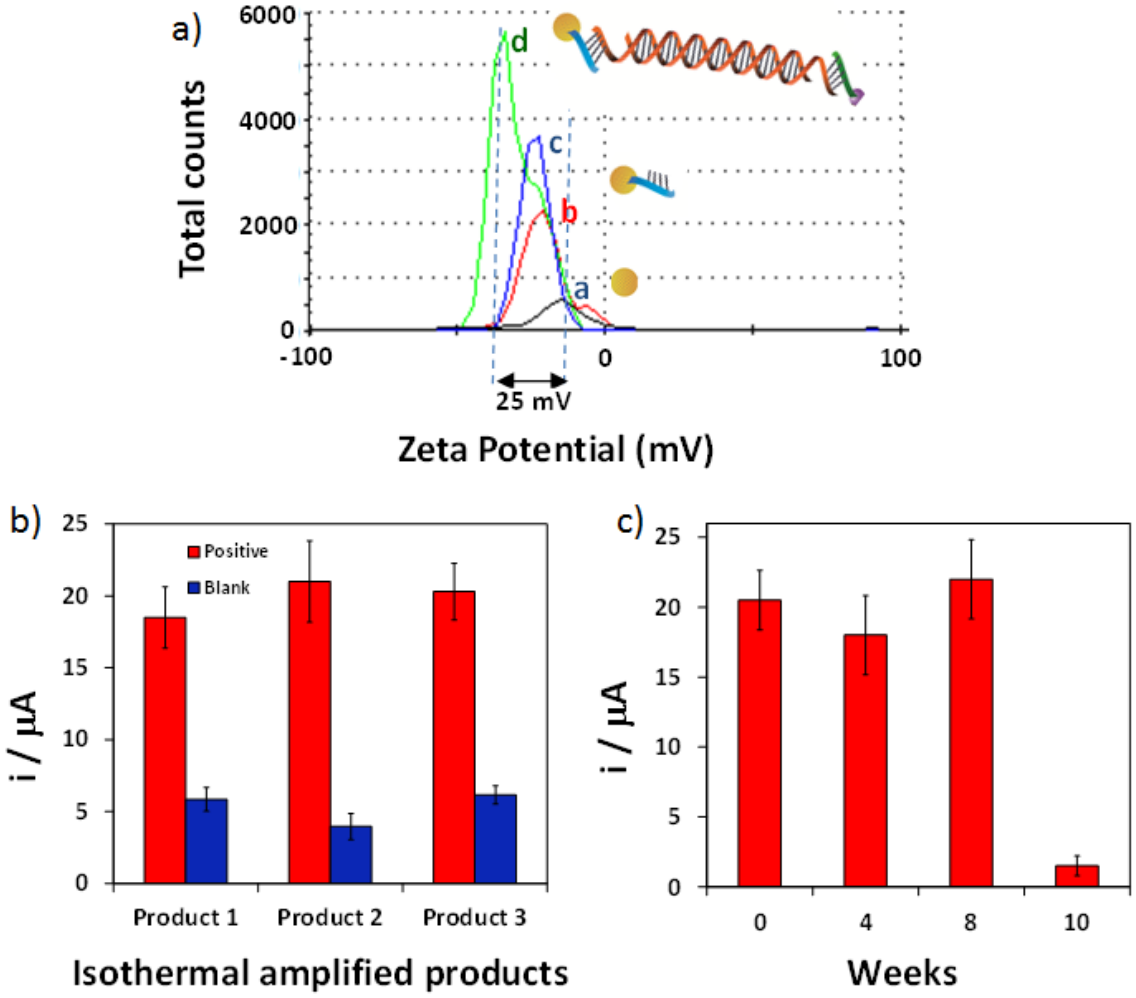

Figure 3. Characterization of AuNP/amplified DNA**. (a)** Diagram for the zeta potential as a distribution versus total counts for a dispersion of AuNPs before (a curve) and after (b curve) the conjugation with the primer and for amplified DNA using the AuNP-labeled primer for a positive (d curve) and a blank (c curve) sample. **(b)** Comparison of the analytical signals obtained for different "positive" and "blank" samples after DNA amplification using AuNP-labeled primers. **(c)** Analytical signals obtained for different isothermal amplifications performed several weeks after the preparation of the conjugate of AuNP/primer.

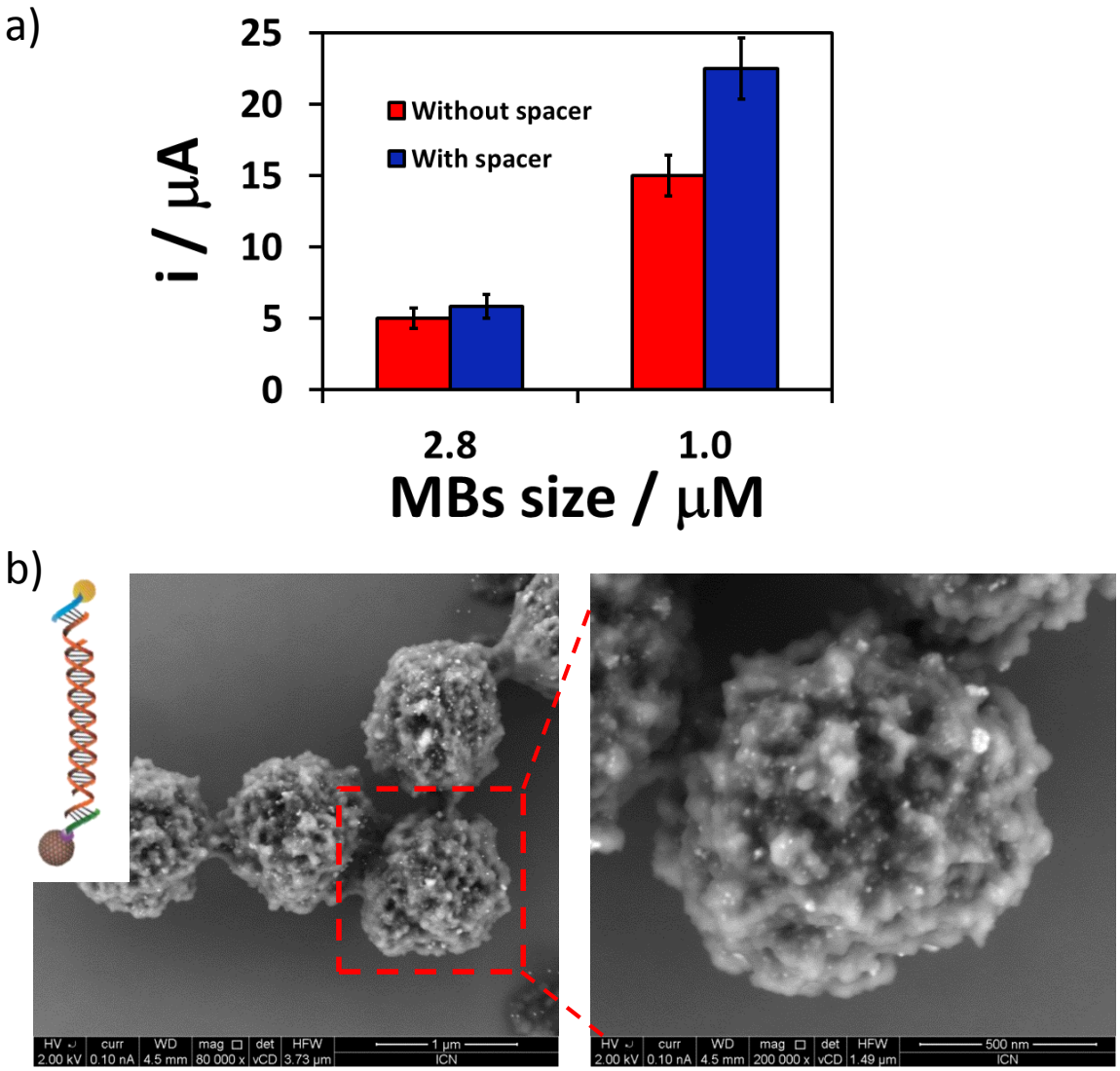

Figure 4. Evaluation of double labeled MB-AuNP isothermal amplified DNA. **(a)** Analytical signals obtained for MBs of two different sizes (2.8 µm and 1 µm) and reverse primers with and without spacer. **(b)** SEM characterization of the MBs after the DNA isothermal amplification. In both cases, forward primers are labeled with AuNPs. Experimental conditions as detailed in the text.

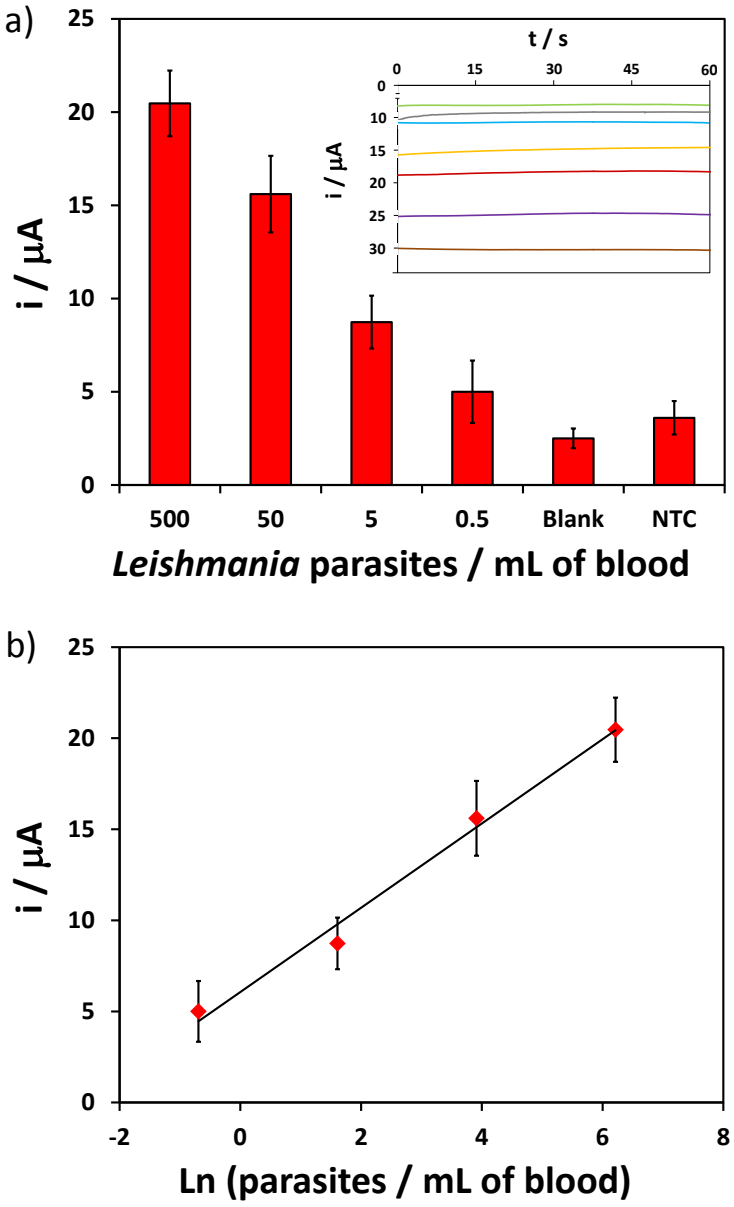

Figure 5. Quantitative *Leishmania infantum* parasite determination. **(a)** Analytical signals obtained for double labeled MB-AuNP isothermal amplified DNA products prepared from different quantities of parasite, after subtracting the background current. Inset graphic corresponds to the chronoamperograms recorded at -1V. Lower current curve (green curve) corresponds to a 1M HCl solution (background signal). **(b)** Logarithmic relationship between the number of *Leishmania* parasites and the value of the analytical signal, after subtracting the background. Experimental conditions as detailed in the text.

A novel methodology for the isothermal amplification of *Leishmania* DNA using labeled primers combined with the advantages of magnetic purification/preconcentration and the use of gold nanoparticle (AuNPs) tags for the sensitive electrochemical detection of such amplified DNA is developed.

Alfredo de la Escosura-Muñiz, Luis Baptista-Pires, Lorena Serrano, Laura Altet, Olga Francino, Armand Sánchez, Arben Merkoçi

Magnetic bead/gold nanoparticle double-labeled primers for electrochemical detection of isothermal amplified *Leishmania* DNA