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A novel triple-lines lateral-flow assay (LFA) with enhanced sensitivity for the detection of *Leishmania infantum* DNA from dog blood samples, taking advantage of the use of gold nanoparticle tags (AuNPs) connected with polyclonal secondary antibodies is developed.
Triple lines gold nanoparticle-based lateral flow for enhanced and simultaneous *Leishmania* DNA detection and endogenous control

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ABSTRACT

A novel triple-lines lateral-flow assay (LFA) with enhanced sensitivity for the detection of *Leishmania infantum* DNA from dog blood samples was designed and successfully applied. The enhanced LFA methodology takes advantage of the use of gold nanoparticle tags (AuNPs) connected with polyclonal secondary antibodies which recognize anti-FITC ones. The polyclonal nature of the secondary antibodies allowed their multiple connections with primary ones, giving rise to the enhancement of the AuNP plasmonics signal. Furthermore, an endogenous control consisting in the amplified dog 18S rRNA gene was introduced so as to avoid false negatives. Using this strategy 0.038 spiked *Leishmania* parasites per DNA amplification reaction (1 parasite/100 μL of DNA sample) were detected, being this detection limit lower than the obtained with other techniques in addition to the advantage of being a universal and simple sensing alternative with interest to be extended to several other biosensing scenarios.

1. Introduction

Diagnostic devices based on low-cost and single-use paper platforms have gained much interest in the last years for their use as ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment free and deliverable to end-users) sensors. [1] The paper chromatography
developed by Martin and Synge at the beginning of 1940s [2] is considered as the first paper-based sensor. Later on, semiquantitative paper-based biosensor for detection of glucose in urine [3] became the most common commercially available point-of-care (POC) lateral flow assay (LFA) device. Initially, the main application of LFAs was as a pregnancy test [4] while nowadays their application is extended to a wide variety of analytes including cancer biomarkers, [5,6] DNA, [7,8] toxins [9,10] and metals. [11,12]

LFAs are characterized by their simple use, rapid result, low cost, good specificity and long shelf life. However, they suffer from analytical performance limitations mainly due to sensitivity and reproducibility issues. In this context, many efforts have been made in order to improve LFA sensitivity by using different alternatives, being of special interest those which take advantage of the tools offered by the nanotechnology. Enhancement of immunosensing approaches based on gold nanoparticles (AuNPs) [13] such as immunogold silver staining, [14,15] dual AuNP conjugates [16] and AuNP loaded with enzymes [17-19] have been reported in the last years. Besides AuNPs, other NP labels such as fluorescent Eu(III) NPs [20] and quantum dots [21] have also been reported. Changes in the paper architecture, [22] the modification of the detection pad with wax-printed pillars [23] and even paper integration into a Lab-in-a-Syringe [24] have also been proposed for improving the performance of LFAs. However, still there is a need for innovative enhancement strategies that would avoid the use of additional modification steps in the whole assay and keep the analytical performance of a simple and cost-effective POC device.

In this context, we present here a very simple and universal enhancement approach based on the use of AuNPs conjugated to polyclonal secondary antibodies able to recognize multiple sites of the primary ones which are targeted to the specific analyte. This novel approach is applied for the detection of *Leishmania* DNA from dog blood samples. Leishmaniasis is a vector-borne and poverty-related disease potentially fatal in humans and dogs, representing an important public health problem. [25,26] Two main *Leishmania* species are associated to several clinical signs: cutaneous leishmaniasis which can be cured but leaves skin damages [27] and visceral leishmaniasis which is mortal if is untreated. [28] According to the World Health Organization, an estimated of 12 million of persons are infected around the world and every year appears 1-2 millions new cases. [29] More than 500,000 cases correspond to visceral leishmaniasis and the mortality estimated is 50,000 deaths per year. [28,29] Canine Leishmaniasis (CanL) caused by *Leishmania infantum* is transmitted by the bite of an insect vector, phlebotomine sand fly, which transmits the flagellated infective promastigote. The intracellular amastigote form is then developed and replicated in the mammal. [30] Different methods for the detection and diagnosis of CanL including parasitological, [31-33] serological [34,35] and molecular techniques [36-42] have been reported. LFA strips for specific CanL antigen (kR39) are commercially available (In BiosInc®, CTK Biotec®, DiaMet IT®) for detection of visceral leishmaniasis [43-46] but there are few examples of detection of amplified (by PCR) CanL DNA using LFA (OligoC-test®), [47] which can enhance in a high extent the sensitivity of the antigen based detection.

Given the importance of *Leishmania* DNA detection we offer a novel LFA design with enhanced sensitivity able to detect very low quantities of DNA amplified analyte. DNA primers labeled with biotin and FITC are used for the DNA amplification reaction so as to obtain FITC/biotin double labeled amplified DNA. The enhanced methodology takes advantage of the use of AuNPs connected with polyclonal secondary antibodies which recognize anti-FITC antibodies. The polyclonal nature of the secondary antibodies allows their multiple connections with primary ones, giving rise to the enhancement of the AuNP signal. Furthermore, an endogenous control consisting in a third line in the LFA that corresponds to the amplification of the 18S rRNA gene is introduced so as to avoid false negatives.

### 2. Experimental

#### 2.1. Chemicals and equipment

Hydrogen tetrachloroaurate (III) trihydrate (HAuCl₄•3H₂O, 99.9%), trisodium citrate (Na₃C₆H₅O₇•2H₂O) and Streptavidin from *Streptomyces avidinii* were purchased from Sigma-Aldrich (Spain). Anti-goat IgG (polyclonal antibody produced in chicken; ab86245) and anti-FITC IgG (polyclonal antibody produced in goat; ab19224) were purchased from Abcam (UK).

All the reagents used for the preparation of the different buffers were supplied by Sigma Aldrich (Spain).

Boric acid (H₃BO₃, 99%) and sodium tetraborate decahydrate (B₄NaO₇.H₂O, 99%) for the preparation of Borate buffer (BB); Sodium phosphate monobasic monohydrate (Na₂HPO₄.H₂O, 99%) and sodium phosphate dibasic (Na₃HPO₄, 99%) and the mortality estimated is 50,000 deaths per year. [28,29] Canine Leishmaniasis (CanL) caused by *Leishmania infantum* is transmitted by the bite of an insect vector, phlebotomine sand fly, which transmits the flagellated infective promastigote. The intracellular amastigote form is then developed and replicated in the mammal. [30] Different methods for the detection and diagnosis of CanL including parasitological, [31-33] serological [34,35] and molecular techniques [36-42] have been reported. LFA strips for specific CanL antigen (kR39) are commercially available (In BiosInc®, CTK Biotec®, DiaMet IT®) for detection of visceral leishmaniasis [43-46] but there are few examples of detection of amplified (by PCR) CanL DNA using LFA (OligoC-test®), [47] which can enhance in a high extent the sensitivity of the antigen based detection.
99%) for the preparation of phosphate buffer (PB); Trizma® HCl (C₄H₁₁NO₃, 99%) and sodium chloride (NaCl, 99.5%) for the preparation of tris buffer saline-tween (TBST); Tween®-20 (C₅₈H₁₁₄O₂₆), sucrose (C₁₂H₂₂O₁₁, 99.5%), bovine serum albumin (BSA, 96%) and sodium dodecyl sulphate (C₁₂H₂₅NaO₄S, 98.5%) for the preparation of blocking buffers; Phosphate buffer saline tablet for the preparation of PBS buffer. All the materials used for the production of the LFIA strips were purchased from Millipore (Billerica, MA 08128, USA): sample and absorbent pads (CFSP001700), conjugate pad (GFPC00080000), detection pad (SHF1800425) and the backing card (HF000MC100). A guillotine Dahle 533 (Germany) was used to cut the strips. An IsoFlow reagent dispensing system (Imagene Technology, USA) was used to dispense the detection and control lines. A strip reader (COZART—SpinReact, UK) was used for quantitative measurements. mQ water, produced using a Milli-Q system (>18.2 MΩ/cm) purchased from Millipore (Sweden), was used for the preparation of all solutions. The stirrer used was a TS-100 Thermo shaker (BioSan, Latvia). A thermostatic centrifuge (Sigma 2-16 PK, Fisher Bioblock Scientific, France) was used to purify the AuNP/antibody conjugates. A high resolution (XHR) SEM (model FEI Magellan™ 400L, Nanolab Technologies, USA) was used for the observation of the AuNPs on the test line of the nitrocellulose strip.

All the size measurements and shape observation of AuNPs were conducted in a Field Emission Gun Transmission Electronic Microscope (model Tecnai™ G2F20, FEI, USA). A spectrophotometer SpectraMax M2e (Molecular Devices, USA) was used to measure the UV-vis spectra of AuNPs suspensions.

2.2. Amplification of Leishmania infantum kinetoplast DNA

Different quantities of promastigote of Leishmania were spiked on DNA samples extracted from negative (previously analyzed by real time PCR [48]) dog blood. Leishmania infantum kinetoplast DNA amplification using reverse and forward primers labelled with biotin and FITC respectively was performed by Recombinase Polymerase Amplification (RPA) isothermal procedure (TwistDx’s®).[49] Samples from dogs without spiked parasite (“blank” samples) were also amplified so as to evaluate the specificity of the method. For endogen control assays, an additional pair of primers that amplify the 18S rRNA gene (always present in both “positive” and “blank” samples), labeled in this case with FITC (forward) and digoxigenin (reverse) was also used.

![Figure 1. Scheme of the enhanced LFA based on the use of secondary antibodies for the detection of double labeled (FITC/biotin) amplified Leishmania DNA (TL1) and 18S rRNA gene (endogenous control) (TL2). CL stands for assay control line.](image-url)
2.3. Preparation of gold nanoparticles

Gold nanoparticles (AuNP) 20 nm sized and stabilized by citrate, were prepared using the Turkevich’s method. [50] Briefly, 50 mL aqueous solution of 0.1% HAuCl₄ was heated to boiling and vigorously stirred in a 250 mL round-bottom flask; 1.25 mL of sodium citrate 1% were added quickly to this solution. Boiling was continued for additional 10 min. The solution was cooled to room temperature with a continuous stirring. The colloids were stored in dark bottles at 4° C. All glassware used in this preparation was previously cleaned in aqua regia overnight and rinsed with double distilled H₂O and reflux was used for all the procedure.

2.4. AuNPs modification with antibodies: preparation of the double antibody solution

The conjugation of AuNPs with antibodies was performed according to the following procedure, previously optimized by our group. [13] First of all, the pH of the AuNPs suspension was corrected to pH 9 with 0.1M borate buffer (BB). Then, 100 µL of a 100 µg/mL anti-goat IgG aqueous solution were added to 1.5 mL of the AuNPs suspension. The resulting solution was incubated for 20 min at 650 rpm. Then, 100 µL of 1 mg/mL BSA aqueous solution were added and the stirring was continued for other 20 min at 650 rpm. Finally, the solution was centrifuged at 14000 rpm and 4°C. The supernatant was removed and the pellet of AuNP/anti-goat IgG was re-suspended in 300 µL of 3 µg/mL anti-FITC IgG solution in BB 2 mM pH 7.4, 10% sucrose. In this way, a suspension containing both anti-FITC IgG and AuNPs/anti-goat IgG was obtained, being immediately used for conjugate pad preparation. TEM micrographs and UV-Vis spectra for AuNPs before and after their conjugation with antibodies are shown at Fig. S1 in the electronic supplementary material (ESM).

2.5. Preparation of the strips

1 mg/mL solution of anti-goat IgG and streptavidin in PB 10 mM, pH 7.4 were spotted onto the detection pad at dispensing rate of 0.05 µL/mm using an IsoFlow reagent dispensing system so as to form the control and test line, respectively. For the detection of the endogen control, an additional line with 1 mg/mL of anti-digoxigenin was also spotted. Then, the detection pad was dried at 37°C for 1h. After that, the membrane was blocked using a 2% BSA aqueous solution for 5 minutes. Finally, the membrane was washed for 15 minutes using PB (5mM pH 7.4), 0.05% SDS and dried at 37°C for 2h. The sample pad was prepared by dipping into 10mM PBS, 5% BSA and 0.05% Tween®-20 and drying at 60°C for 2h. The conjugate pad was prepared dipping it into the previously prepared double antibody solution (anti-FITC IgG and AuNP/anti-goat IgG) and drying under vacuum for 1 h. The different pads were sequentially laminated 2 mm with each other and pasted onto the adhesive backing card in the following order: detection, conjugation, sample and absorbent pads. Finally, the strips were cut 7 mm wide and stored in dry conditions at 4°C until their use up to a week.

2.6. Lateral-flow assay procedure

For the evaluation of the signal enhancement, amplified *Leishmania* DNA sample solutions prepared from dog blood samples spiked with a fixed quantity of 20 parasites were diluted in Tris buffer saline 10mM Trizma®-HCl pH 7.6 and 0.05% Tween®-20 (TBST) at different dilution factors. Typical assay consisted in mixing 10 µL of the diluted sample (different dilution factors in TBST were assayed: 1:100, 1:250: 1:500 and 1:1250) with 200 µL of TBST, immersing the strip into this solution and keeping for 10 min until the flow is

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**Figure 2.** Optimization of the enhanced lateral-flow assay. (A) Effect of running buffer on an amplified *Leishmania* product diluted at 1:50. Buffers are: (a) 25mM Tris, 150 mM NaCl, pH 7.6, (b) 50mM Tris, 138mM NaCl, 0.27mM KCl, 1% BSA and (c) 50mM Tris, 150 mM NaCl, 0.05% Tween-20. (B) Effect of primary antibody concentration in double antibody-AuNP conjugate. Assays performed for an amplified *Leishmania* DNA product diluted at 1:100. Other conditions as detailed at the experimental section.
stopped. Then 200 μL of TBST were dispensed in order to wash away the excess of the double antibody solution. For the quantitative *Leishmania* parasite determination, concentrated amplified DNA (without any dilution) coming from dog blood samples spiked with different quantities of parasite (0.04, 0.08, 0.4, 1, 2, 4 and 20 parasites) were evaluated. Three replicates of each sample were done and intensities of lines were read with the strip reader so as to obtain the corresponding calibration curve.

### 3. Results and discussion

#### 3.1 Principle of the enhanced detection

The principle of the signal enhancement is based on an indirect ELISA. In a typical assay, after the analyte recognition by a capture antibody, the sandwich is completed with a label-free primary antibody. After that, an AuNP labeled polyclonal secondary antibody is used for the recognition of the primary one. The polyclonal nature of the secondary antibody makes possible the connection of a high number of labeled antibodies to each primary one, giving rise to an increase in the number of AuNP labels and consequently an enhancement in the analytical signal. [51]

In our approach, the novel and simple strategy for the signal amplification is based on the immobilization on the conjugation pad of primary antibodies specific to the analyte and secondary antibodies labeled with AuNPs which recognize the primary ones. The secondary antibodies (chicken anti-goat IgG) were labeled with AuNPs which can recognize a primary one that acts as detection antibody (goat anti-FITC) forming a complex which was dispensed onto the conjugation pad. Two reagents are deposited on the nitrocellulose membrane: streptavidin and anti-goat IgG, forming the test line (TL1), and the assay control line (CL) respectively. When the sample starts to flow through the strip, the primary antibody/secondary antibody complex is released, causing the capture of the amplified DNA through the tails labeled with FITC. While the sample keeps migrating though the membrane, the test line becomes visible due to the binding of streptavidin with the biotin present in the amplified DNA. The control line turns visible when the anti-species antibodies capture any excess of antibody labeled with gold nanoparticles (see Figure 1).

Using the standard LFA design, a negative response (no signal in the detection line) can be due not only to the absence of *Leishmania* in the sample but also to errors in the DNA extraction or in the DNA amplification procedure. In order to solve this, an endogenous control was also performed and introduced in the LFA design. It consists in introducing an additional pair of primers that amplify the 18S rRNA gene (always present in the sample), so this amplified DNA must be always present, for both positive and blank samples. These primers were labeled with FITC and digoxigenin as schematized in Figure 1. As result, in the positive samples both products (biotin/FITC and digoxigenin/FITC labeled) will be present while only the digoxigenin-FITC pair will be found in the blank samples.

**Figure 3.** Evaluation of the signal enhancement. (A) Pictures of LFA strips after the assay performed for amplified *Leishmania* DNA at different dilution factors, containing the endogenous control. The direct and the enhanced assays are also compared. TL1 corresponds to the test line while TL2 stands for the endogenous control. (B) The corresponding intensity values (TL1) obtained with the strip reader for both direct and enhanced assays.
In order to detect both products, the LFA strip was slightly modified, adding an additional line in the detection pad (TL2), where anti-digoxigenin antibodies were immobilized. This line must be always visualized, even for blank samples, evidencing that the DNA amplification procedure worked properly, as illustrated in Figure 1.

3.2 Optimization of the enhanced lateral flow assay

Different parameters affecting the analytical signal, including blocking steps, concentration of antibodies in the double antibody complex and running buffer were optimized. In order to minimize reagents consumption, this optimization was performed for assays without the endogenous control, so only the test line (TL1) and assay control line (CL) were visualized.

Blocking agent. Nitrocellulose membranes have been traditionally blocked with different agents (e.g. proteins, surfactants or polymers) not only to avoid non-specific bindings of nanoparticles over the membrane [52,53] but also to control the flow rate and stabilize test and control line. [54] The best conditions for blocking the membrane for each specific approach must be thoroughly optimized. In our case, a solution containing 2% BSA for preventing the non-specific adsorptions after dispensing the antibodies over the nitrocellulose membrane was found as suitable as blocking agent (see Figure 2A).

Running buffer. The formation of antigen-antibody complex can be affected by matrix parameters such as pH, temperature and ionic strength. [55] Tris buffer is well known for its capability to solubilize DNA so as to avoid its degradation, so three running buffers with different Tris concentrations and additives were tested, for positive and blank samples as shown in Figure 2A. Results showed that when the ionic strength of running buffer increased, the test line became more intense, the sensitivity was improved and no unspecific signal in the test line for the blank sample was observed. Although when the ionic strength between buffers was not significantly different, the presence of surfactant (Tween-20®) allowed a better flow of nanoparticles through the membrane avoiding non desirable adsorptions. Thus, the most suitable running buffer for this assay was found to be TrisHCl 50 mM, 150mM NaCl and 0.05% Tween-20®. These observations are in agreement with previously reported studies showing that ionic strength of running buffer together with the presence of surfactant have an important influence of the background signal decreasing the non-specific bindings over the membrane. [56,57]

Concentration of primary antibody. Different concentrations of primary antibody for a fixed quantity of the conjugate of the secondary antibody/AuNPs were evaluated. It was found that the most suitable concentration of goat anti-FITC in the double antibody solution was 3 µg/mL (see Figure 2B). This can be probably due to the fact that for higher concentrations of primary antibody, the primary Ab / secondary Ab ratio is close to 1:1 and consequently the AuNPs concentration in the test line is lower, as also schematized in Figure 2B.

Figure 4. SEM characterization of the signal enhancement. SEM images of the test line (TL1) of nitrocellulose strips after a direct (A) an enhanced (B) LFA performed for an amplified Leishmania DNA product diluted at 1:100. Pictures on the left belong to the corresponding strips. Arrows indicate areas with high density of AuNPs.
3.3 Semiquantitative assay: evaluation of the signal enhancement

Preliminary tests for amplified DNA using the enhanced LFA strategy were performed under the optimized conditions. The results were compared with those obtained using a direct LFA without using secondary antibodies and summarized in Figure 3. In both cases, the concentrated amplified DNA was serially diluted so as to evaluate the ability of the sensing system to detect lower quantities of labeled product. As also shown in Figure 3A, the additional test line (TL2) corresponding to the endogenous control is clearly visualized. Furthermore, the intensity of color in the control line remains constant while the ones corresponding to the two test lines decrease when the dilution factor is increased, demonstrating that the DNA amplification procedure worked properly. A clear improvement in the sensitivity thanks to the use of secondary antibodies was noticed even with the naked eye.

The obtained enhancement allows visual detection of amplified DNA diluted at a 1:500 factor, whereas with the direct assay only products diluted up to 1:250 are visualized. The advantages of the enhanced strategy are even more evident with the measurements of optical density of the test line (TL1) performed with a scanner (Figure 3B).

It is important to point out that with this novel strategy not only the sensitivity is improved but also the lines are better defined, which is crucial for an adequate and reproducible reading by the scanner.

Thanks to the optical density measurements, amplified DNA diluted up to a 1:1250 ratio can be detected, ensuring that false negatives are avoided by the visualization of the endogenous control line (TL2).

The enhanced methodology was also evaluated through the examination by scanning electron microscopy (SEM) of the test line (TL1) of nitrocellulose strips after LFAs performed for a fixed quantity of amplified Leishmania DNA, as shown in Figure 4. For the direct assay, a small amount of dispersed AuNPs is found on the surface of the strip, observed as discrete spots (Figure 4A). However, in the case of the enhanced assay, a much higher density of AuNPs is observed, as evidenced in Figure 4B. These observations are in agreement with the proposed enhancement principle, suggesting that the use of secondary antibodies highly increases the density of AuNPs in the test line, leading to an important enhancement in the optical density and consequently in the sensitivity of the assays.

**Figure 5.** Quantitative Leishmania infantum parasite determination. (A) Pictures of LFA strips for an enhanced lateral flow assay containing the endogenous control. TL1 corresponds to the test line while TL2 stands for the endogenous control. (B) Corresponding bar intensity graphic (left) and logarithmic relationship between the number of Leishmania parasites and the % of intensity in the test line (right).
3.4 Quantitative *Leishmania infantum* parasite determination

The enhanced strategy in combination with the endogenous control was applied for the evaluation of amplified DNA prepared from samples containing different quantities of spiked parasite. As shown in Figure 5A, a gradual increase in the intensity of test line (TL1) was observed for increasing amounts of parasite, being possible to detect up to 0.08 parasites with the naked eye. As expected, the intensity of the line corresponding to the endogenous control (TL2) remains almost constant since the amount of 18S rRNA gene (from dog DNA) is the same in all cases, independently of the quantity of parasites spiked in the sample. These evidences are also corroborated by the measurements performed with the scanner (Figure 5B, left).

Values of intensity of test line (TL1) vs. the logarithm of number of parasites were plotted (Figure 5B, right). A linear relationship between the parasite concentration and the intensity of the test line in the range 0.04 to 20 spiked parasites was obtained. A limit of detection (LOD) of 0.038 parasites per DNA amplification reaction (1 parasite/100μL of DNA) was estimated, as the parasite number giving a signal equal to the blank signal plus three times its standard deviation. The reproducibility of responses (n=3) for 2 spiked parasites was also studied, obtaining a relative standard deviation (RSD) of 4%.

The LOD obtained is almost the same as the one obtained with only one test line for *Leishmania* parasite detection (see the study performed without endogenous control at the Fig. S2 and Fig. S3 in the ESM), demonstrating that the addition of a second test line provides in the same lateral flow strip a control for checking the DNA amplification and also a tool for *Leishmania* parasite detection without affecting the sensitivity of the enhanced LFA.

Most of LFA for *Leishmania* detection are offered for qualitative tests only being few of them able to achieve semi-quantitative analytical response. [39,44,58,59] Our AuNP based amplification approach results are quite similar to those obtained by semi-quantitative test using nucleic acid sequence based amplification (NASBA) and coupled to oligochromatography (OC) for *Leishmania* detection [60] and even better than the 1 parasite per PCR detection limit offered by the OligoC-test®. [47] Furthermore, our technique provides a valuable proof of concept due to the enhancement approach which is a universal methodology that can be applied for any LFA design.

4. Conclusions

A novel design of Lateral Flow Assay (LFA) based on the use of secondary antibodies in the conjugate pad was successfully accomplished and applied for the detection of amplified *Leishmania infantum* DNA extracted from dog blood. The use of labeled primers allows to obtain double labeled (FITC/biotin) products that can be detected in a LFA. The polyclonal nature of the secondary antibodies allows their multiple connections with primary ones, giving rise to the enhancement of the AuNP signal in the test line and consequently the sensitivity of the assay is highly increased, allowing to detect up to 0.038 parasites per DNA amplification reaction (1 parasite/100μL of DNA).

Furthermore, an additional control, the so-called endogen control, was included so as to avoid false negatives. It was simply performed by adding an additional pair of primers that amplify the 18S rRNA gene (always present in the sample) labeled with FITC and digoxigenin and introducing an additional test line in the LFA strip, containing anti-digoxigenin antibodies. This approach was successfully implemented, without losing the efficiency of the signal enhancement. The proposed enhancement strategy is a versatile and universal methodology that can be applied for any LFA design. It also has the advantage of the fact that the specific antibody against the analyte does not need to be directly labeled, representing clear advantages in terms of technology cost.

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Electronic Supplementary Material: Supplementary material (characterization of AuNPs and study of the performance of the system with only one test line for Leishmania parasite detection) is available in the online version of this article at http://dx.doi.org/10.1007/s12274-***.***
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