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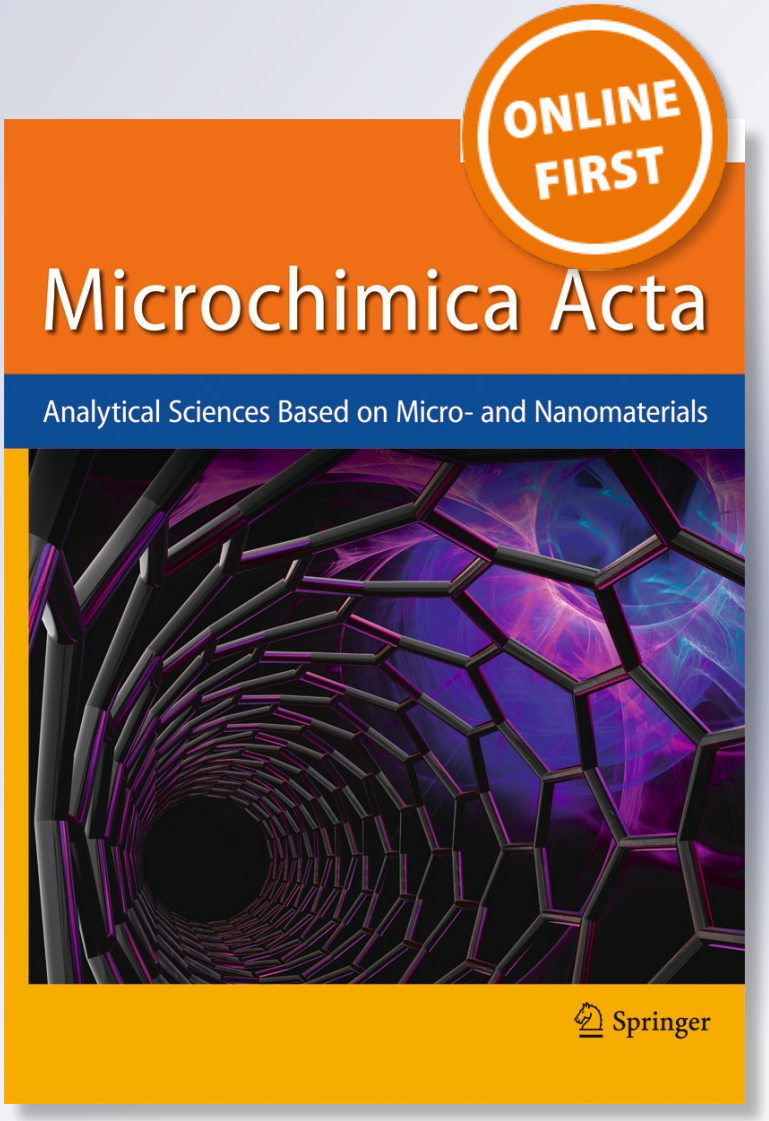

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ORIGINAL PAPER

An electrochemical immunosensor for anti-*T. cruzi* IgM antibodies, a biomarker for congenital Chagas disease, using a screen-printed electrode modified with gold nanoparticles and functionalized with shed acute phase antigen

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Abstract The authors describe a sandwich immunosensor for anti-IgM *T. cruzi*-specific antibodies which are a biomarker for congenital Chagas disease (CCD). A screen-printed carbon electrode (SPCE) was modified with gold nanoparticles (AuNPs) and then functionalized with recombinant shed acute phase antigen (SAPA). The AuNPs/SPCE was characterized by scanning electron microscopy, cyclic voltammetry, and X-ray diffraction. The AuNPs provide an increased active surface area, high conductivity, and improved electrocatalytic characteristics, this resulting in a significant improvement of the detection limit. Following interaction between immobilized SAPA and the target antibodies in the serum sample, secondary antibody (an anti-human IgM-HRP conjugate) and solutions of H_2O_2 and 4-tert-butylcatechol were added and the enzymatic product was detected at −100 mV (vs. Ag/SPCE). The resulting current was proportional to the anti-*T. cruzi* IgM antibodies present in the sample and showed a linear trend from 10 to 200 ng mL^{-1} (R = 0.998). The detection limit was 3.03 ng mL^{-1}. These values demonstrate that this electrochemical immunosensor can be used as an alternative serological method for diagnosis of congenital Chagas disease.

Keywords Sandwich immunoassay · Recombinant antigen · Scanning electron microscopy · Cyclic voltammetry · X-ray diffraction · Horseradish peroxidase · Serology

Electronic supplementary material The online version of this article (doi:10.1007/s00604-016-1752-4) contains supplementary material, which is available to authorized users.

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Introduction

Chagas disease (CD) is a zoonotic infection caused by the hemoflagellate *Trypanosoma cruzi* (*T. cruzi*). The infection is endemic in rural areas of Latin America but affects approximately 7 to 8 million people worldwide [1]. Untreated patients with this pathology develop cardiac abnormalities including myocardial injury, cardiac dilation, arrhythmia and severe conduction alterations; gastrointestinal tract disorders characterized by megaesophagus and megacolon or mixed forms of this disease [2, 3].

Increased travelling and mass migration of asymptomatic and chronically-infected individuals from endemic areas have caused the infection to be disseminated all over the world [4, 5]. The impact of CD in non-endemic areas (Europe, North America, Japan, and Australia) occurs mainly by non-vectorial transmission, particularly; congenital infection, blood transfusions, and organ transplants [6, 7].

In 2004, the Pan American Health Organization focused its attention on the congenital transmission of CD and organized a specific consultation [8, 9]. The advisory group emphasized that congenital transmission constituted the main and most persistent form of the parasitosis among within the human population.

Although congenital transmission of CD may occur during any phase of maternal disease, transmission during the first trimester of pregnancy is probably rare because maternal blood supply becomes continuous and diffuse in the entire placenta only after the 12th week of gestation. Therefore,

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transmission of blood parasites is more likely to occur during the second and third trimesters of pregnancy (prenatal transmission) and during labor (perinatal transmission) through placental breaches/tears [10, 11]. To control transmission, it is essential to test all pregnant women living in endemic countries and all pregnant women having migrated from, or having lived in, endemic countries [12, 13].

Currently, only direct parasite detection tests are able to confirm infection at birth. The serological profile in the first month of the acute phase of CD corresponds to a classical primary immune response. Specific immunoglobulin M (IgM) appears early in the acute phase of *T.cruzi* infection and can be used in the diagnosis of congenital transmission [14]. However, after 9 months of age (with the disappearance of maternal antibodies), conventional immune-globulin G (IgG) serology may allow the diagnosis of congenital infection [15, 16]. The requirement of long-term follow-up on most infants to confirm whether or not they are infected often causes decreases of the adherence to therapy, with a consequent lack of opportunity for almost 70 % of new born to be diagnosed and treated in a timely fashion [17, 18].

Conventional serological tests for CD usually employ semi-purified antigens from the epimastigote form of *T. cruzi*. Consequently, CD tests yield relatively large numbers of inconclusive and false positive results [19, 20], mainly when a concomitant infection, such as leishmaniasis, is present. Additionally, the sensitivity of CD tests is far from ideal in the diagnosis of the early acute phase of the disease or in patients with low titers of anti-*T. cruzi* antibodies.

To overcome these problems, several laboratories have developed new serological diagnostic tests using antigens from infective trypomastigote forms or a combination of *T. cruzi* recombinant proteins and/or synthetic peptides [21, 22]. Among them, the SAPA antigen (for Shed Acute Phase Antigen) has been used in several studies and has shown to be a good marker for the diagnosis of *T. cruzi* infection.

This antigen has been used to analyze the reactivity of antibodies occurring in sera of chronic chagasic mothers and their newborns [23, 24] and it is known to react with IgM and IgG antibodies of sera from both acute and chronic chagasic patients [25].

Anti-SAPA antibodies have been detected in 90 % of acute chagasic patients and in 7–10 % of chronic patients. Because SAPA reacted with fetal IgM and IgG antibodies present in the cord blood from newborns infected with *T. cruzi*, it has been suggested that the detection these antibodies could be used to distinguish congenitally infected infants from uninfected infants [26, 27].

One additional drawback affecting most serological methods currently used to diagnose CD is that they are not suitable for automatic instruments, a desirable feature for blood banks, where a large number of samples must be screened for this disease.

Electrochemical methods have attracted increasing attention because of their high sensitivity, fast response, simplicity, low instrumental costs, small sample volumes, and portability. Among the many electrochemical systems that can be applied for diagnostic purposes, the combination of amperometry (A) and screen-printed carbon electrodes (SPCE) can add mass production capabilities and represents one of the most convenient alternatives [28, 29].

Moreover, the surface of the SPCE is amenable for modification by electrodeposition with a variety of metallic nanoparticles (NPs) such as copper, gold, platinum, palladium, or silver. Most of these modifications can provide increases in surface area and render sensors with enhanced limits of detection and improved electrocatalytic characteristics [30, 31].

Considering these factors, we report the development of an electrochemical immunosensor based on AuNP/SPCE and applied to the quantification of anti-Igmt. *cruzi* specific antibodies, used as biomarkers for CCD.

Experimental

Reagents and solutions

All reagents used were of analytical reagent grade were used without further purification. 3-Mercaptopropionicacid (MPA), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), DL-dithiothreitol (DTT), $HAuCl_4$ 0.01 %, N-2-hydroxyethylpiperazine-N-2-ethanesulfonicacid sodium salt (HEPES), and 4-tert-butylcatechol were purchased from Sigma-Aldrich (St. Louis, MO, USA, http://www.sigmaaldrich.com). Anti-IgM–mouse monoclonal Ab (1000 $mgmL^{-1}$) and HRP-conjugated anti-IgM–Ab (1000 $mgmL^{-1}$) were purchased from Abcam (USA, http://www.abcam.com). H_2O_2 was purchased from Merck (USA, http://www.merck.com). Aqueous solutions were prepared using 18 MΩ.cm water from a Milli-Q system.

Instruments

Amperometric measurements and voltammetric analysis were performed using a BAS LC-4C potentiostat and a BAS 100 B/W Electrochemical Analyzer (BioAnalytical System, West Lafayette, IN, http://www.basinc.com), respectively. A SPCE made up of three electrodes was used. A silver ink as pseudo-reference electrode, a graphite ink as auxiliary electrode and, a graphite ink circular (Ø = 3 mm) with and without modifications as working electrode were used for all the measurements. All potential measurements were compared vs. Ag/SPCE. Absorbance was determined using a Bio-Rad Benchmark

microplate reader (Japan, http://www.bio-rad.com) and a Beckman DU 520 general UV/vis spectrophotometer (http://www.beckmancoulter.com).

All pH measurements were made with an Orion expandable ion analyzer (Orion Research Inc., Cambridge, MA, USA) Model EA 940 equipped with a glass combination electrode (Orion Research Inc., http://www.bioesanco.com.ar).The structures and composition of the nanoparticles were determined by X-ray diffraction (XRD) using a Rigaku D-MAX IIIC diffractometer using copper radiation (ka = 0.154178 nm) and containing a nickel filter. The morphology of the electrosynthesized nanoparticles was investigated using a LEO 1450VP scanning electron microscope (SEM, http://labmem.unsl.edu.ar).

Recombinant antigen

The recombinant *T. cruzi* Shed-acute-phase-antigen, SAPA, (aa 61–460, Swiss-Prot # Q00773, expressed in *E. coli*) was purchased from Bioclone Inc. (USA).

Reference sera

To obtain the human anti-*T.cruzi* reference sera, a previously-described procedure [33] was performed, following the herein described modifications. Serum samples were initially obtained from children born from seropositive mothers who were confirmed for Congenital Infections by the detection of blood parasites, by direct parasitological methods/thin or thick blood smears, the Strout method, and buffy coat on slide. These samples were collected by trained personnel according to established protocols and without personal identifiers.

In the first step, an anti-*T.cruzi* serum was obtained. For this, SAPA coupled to 3-aminopropyl-modified controlled pore glass (AP-CPG, previously activated with 5 % aqueous solution of glutaraldehyde) were transferred into a column, in which the serum sample was added. After washing all non-adsorbed serum proteins, a solution containing specific anti-*T. cruzi* antibodies was obtained.

After that, a second immune adsorption procedure was carried out to obtain a reference serum containing only IgM anti-*T. cruzi* antibodies. In this case, AP-CPG was coupled with anti human IgM antibodies, packed into a new column, and put in contact with the eluted obtained in the first immune adsorption procedure.

The eluted solution (containing only IgM anti-*T. cruzi* antibodies) was analyzed using Quantitative Human IgM ELISA and resulted in a concentration of anti-*T. cruzi* IgM antibodies of 155 ng mL^{-1}.

Modification of SPCE electrode

Preparation of SPCE

An electrode pretreatment was carried out before the electrodeposition procedure to oxidize the graphite impurities and to obtain a more hydrophilic surface, with the aim of improving the sensitivity and reproducibility of the results. The graphite electrode surface was pretreated by applying a potential of +1600 mV for 120 s and of +1800 mV (vsAg-SPCE) for 60 s in 5 mL of 0.25 mol L^{-1} acetate buffer, containing 0.01 mol L^{-1} KCl pH 4.75, under stirred conditions. Then, the electrode was washed using 0.01 mol L^{-1} phosphate buffered saline (PBS) pH 7.20 and stored in the same buffer at 4 °C.

Synthesis of SAPA–MPA–AuNP/SPCE

For the electrodeposition of AuNPs, the SPCE was immersed in a solution containing 0.01 % $HAuCl_4$ and 0.1 mol L^{-1} KNO_3, as supporting electrolyte. Then, a constant potential value of −200 mV vs. Ag was applied for 60 s. Next, the modified electrode was rinsed by stirring (at 250 rpm for 30 s) in purified water and finally carefully dried undera N_2 stream. The AuNP/SPCE was then characterized by cyclic voltammetry.

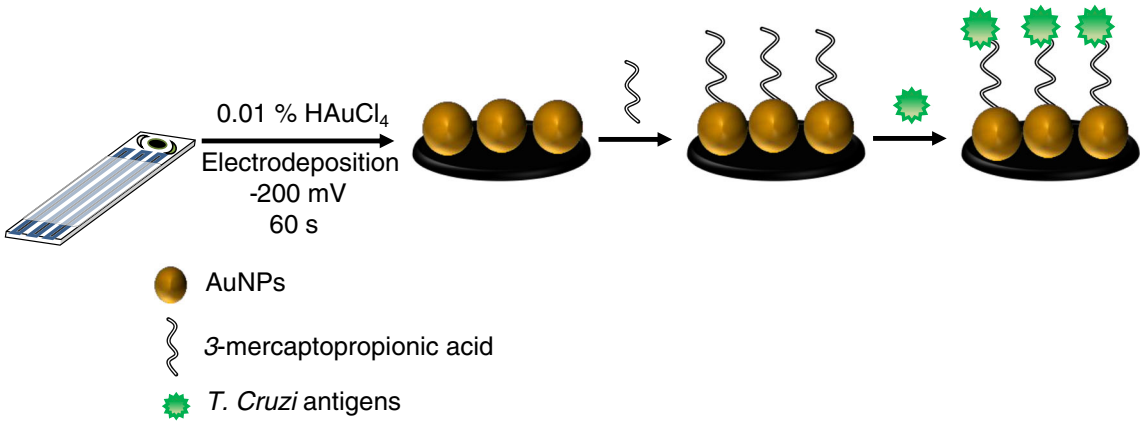
In order to immobilize the SAPA, the AuNP/SPCE was immersed in a solution containing 0.04 mol L^{-1} MPA in EtOH/H_2O (75/25, *v*/v) for 15 h, at room temperature. Here the –SH group of MPA reacted with the surface of AuNPs, exposing–COOH groups, through which the immobilization of SAPA proteins can be done. The resulting MPA–AuNP/SPCE was rinsed several times with purified water and dried under a N_2 stream.

Finally, SAPA was immobilized on the MPA–AuNP/SPCE (Scheme 1) by first activating the–COOH groups of MPA by rinsing the electrode surface with a solution containing 0.01 mol L^{-1} EDC-NHS in 0.05 mol L^{-1} PBS pH 7.20. The electrode was then rinsed with purified water and immersed in a solution containing SAPA (10 μg mL^{-1}), overnight at 4 °C.

T. cruzi specific IgM antibodies measurement procedure

The procedure for this analysis is summarized in Table 1. Briefly, the SAPA–MPA–AuNP/SPCE was conditioned with 0.01 mol L^{-1} PBS pH 7.20 for 3 min. Next, the unspecific binding was blocked by immersing the electrode in a solution containing 1 % bovine serum albumin in 0.01 mol L^{-1} PBS pH 7.20 by 5 min at room temperature.

The excess protein was removed by rinsing the surface with 0.01 mol L^{-1} PBS pH 7.20 for 3 min. Second, a serum



Scheme 1 Modification procedure for obtaining SAPA–MPA–AuNP/SPCE

sample, previously diluted 100-fold with 0.01 mol L^{-1} PBS pH 7.20, was dispensed on the electrode surface an allowed to stabilize for 5 min. The electrochemical immunosensor was then washed several times with 0.01 mol L^{-1} PBS pH 7.20.

The anti-human IgM–HRP conjugate (dilution of 1/2000 in 0.01 mol L^{-1} PBS pH 7.20) was then placed on the electrode 5 min. The immunosensor was then washed free of any traces of unbound enzyme conjugate. Finally, the substrate solution (1 mmol L^{-1} H_2O_2 and 1 mmol L^{-1}4-tert-butylcatechol in 0.01 mol L^{-1} phosphate–citrate buffer pH 5.05) was added to the SAPA–MPA–AuNP/SPCE and the enzymatic product was detected at −100 mV.

The resulting cathodic current was found to be proportional with respect to the IgM anti-*T. cruzi* antibodies concentration present in the sample (Scheme 2).

In order to ensure selectivity, the electrochemical measurement procedure described above was performed on: reference sera, negative controls, samples and blank (0.01 mol L^{-1} PBS pH 7.20). Before each sample analysis, the electrochemical immunosensor was exposed to a desorption buffer (0.1 mol L^{-1} glycine-HCl pH 2.00) for 5 min and then washed with 0.01 mol L^{-1} PBS pH 7.20.

With this treatment, the immune complex bound to the immobilized antigen was desorbed, allowing starting with the next determination. The storage of the device was made in 0.01 mol L^{-1} PBS pH 7.20 at 4 °C.

Results and discussion

Characterization of AuNP/SPCE surface

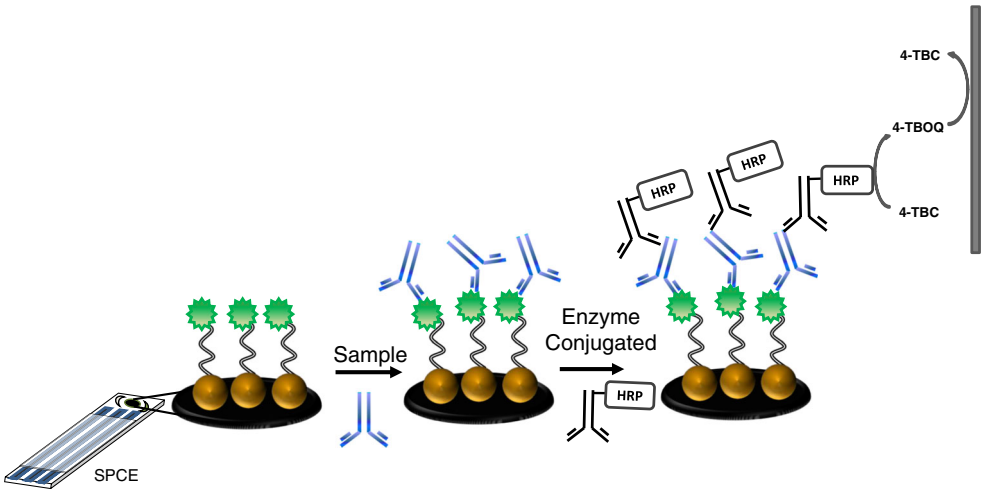
Figure 1 shows the SEM images of the surface of the SPCE before (a) and after the modification with AuNPs (b). As it can be observed, a uniform layer of AuNP with a diameter ranging from 20 to 50 nm was obtained. The crystalline structure of the AuNPs, obtained by XRD is shown in Fig. 1c, where distinctive features can be identified. The peak at 2 (26.3) was attributed graphite and the peaks at 2 (42.6, 54.3 and 68.91) was attributed to the AuNPs. In line with the SEM image, the average size of the crystalline structure of the deposited AuNPs was calculated to be approximately 30 nm (according to the Scherrer formula, t = K λ/B cos).

The electrochemical behavior of 4-tert-butylcatechol was examined by cyclic voltammetry. Figure 1d shows the cyclic voltammogram recorded in 1 mmol L^{-1}4-tert-butylcatechol in

Table 1 Sequences required for the *T. cruzi* specific IgM antibody immunoassay

Sequence	Conditions	Time
Blocking solution	1 % albumin in 0.01 mol L^{-1} PBS pH 7.20	5 min
Washing buffer	0.01 mol L^{-1} PBS pH 7.20	3 min
Serum samples	Diluted 100-fold	5 min
Washing buffer	0.01 mol L^{-1} PBS pH 7.20	3 min
Enzyme conjugated	HRP-conjugated (dilution of 1/2000)	5 min
Washing buffer	0.01 mol L^{-1} PBS pH 7.20	3 min
Substrate	5 μL 1 mmol L^{-1} H_2O_2+ 1 mmol L^{-1}4-TBC in phosphate–citrate buffer, pH 5.05	1 min
Signal analysis	LC-4C amperometric detector, −100 mV	1 min

Scheme 2 Principle of immune reaction



0.01 mol L^{-1} phosphate–citrate buffer pH 5.05 from −300 to 800 mV (Scan rate 50 mV s^{-1}) for the unmodified SPCE (tracea) and the AuNP/SPCE (curve b). As expected, the electrode modified with AuNP exhibited the characteristic increase of the anodic and cathodic peaks, thus confirming the successful modification process.

Larger peak currents and a smaller peak-to-peak potential separation (ΔE) were observed at the AuNP/SPCE (voltammogram b; I_{pa} = 25.37 μA, I_{pc} = 22.97 μA; ΔE = 245 mV) when compared with the bare SPCE (voltammogram a; I_{pa} = 20.26 μA, I_{pc} = 17.95 μA; ΔE = 265 mV). The former observation was attributed to the enhanced electrochemical activity of the gold nanoparticles, which allowed the increase of the electrode active area.

The latter observation can be also associated with the presence of the AuNP, which yielded slight shifts of anodic peak potential to less positive values, giving rise to a smaller peak-to-peak separation (ΔE = 245 mV). This fact suggests as light improvement in the electrocatalytic properties the electrode produced by the addition of the AuNP, which facilitated the electron-transfer process.

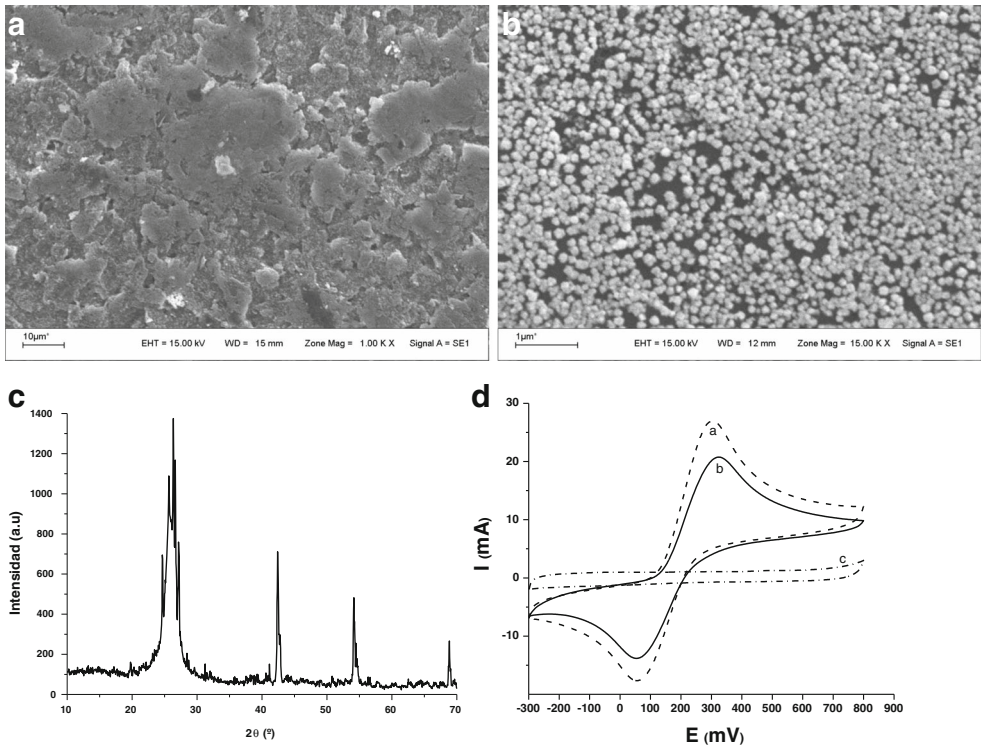


Fig. 1 **a** SEM image of unmodified SPCE. **b** SEM image of SPCE surface modified with electrodeposited AuNPs. **c** XRD pattern of AuNP/SPCE. The peak at 2 (26.3) was attributed graphite and the peaks at 2 (42.6, 54.3 and 68.91) was attributed to the AuNPs. **d** Cyclic voltammograms of 1 mmol L^{-1}4-tert-butylcatechol in 0.01 mol L^{-1} phosphate-citrate buffer pH 5.05 from −300 to 800 mV (Scan rate 50 mV s^{-1}) in unmodified SPCE (curve b), modified with electrodeposited AuNPs (curve a), and background (curve c)

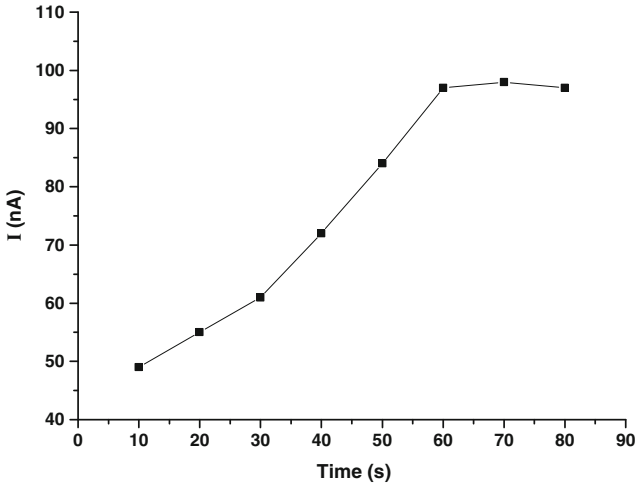


Fig. 2 Study of electrodeposition time from 10 to 80 s

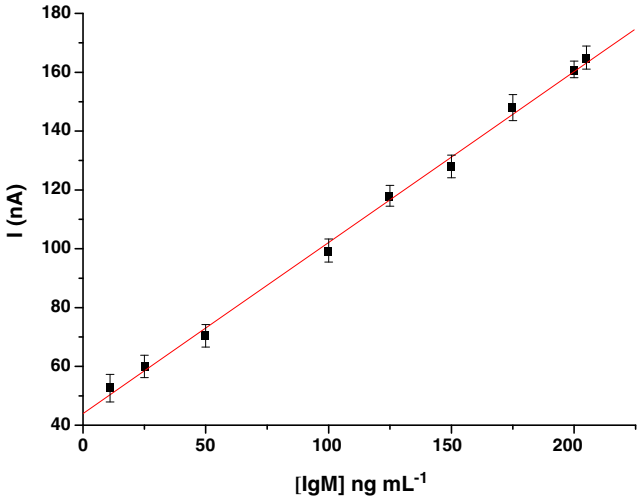


Fig. 4 Calibration plot obtained by plotting *i* versus anti-*T. cruzi* IgM antibody concentration. A linear relation, ΔI (nA) 43.99 + 0.581 C IgM antibodies, was observed between *i* and IgM concentrations in the range from 10 to 200 ng mL^{-1}

Optimization of electrodeposition time and potential (t_{edep} and E_{edep}) of AuNP/SPCE

As already described above, the electrodeposition of AuNP on the electrode surface was strongly affected by several parameters, such as the t_{edep} and E_{edep}. Both factors have been optimized to obtain the best analytical performance in our device.

For the optimization of the t_{edep} the potential used was −200 mV and the electrodeposition time was evaluated in a range of 10–80 s. As Fig. 2 shows, increasing the electrodeposition time from 10 to 60 s lead to significant current increases an effect that plateaued at longer deposition times. Therefore, an electrodeposition time of 60 s was selected as optimum.

The effect of the E_{edep} was investigated using a deposition time of 60 s and varying the potential applied to the working electrode in the-50 to-250 mV range. As shown in Fig. 3, significant increases in the current were obtained as the potential applied was changed from −50 mV to −200 mV. Because no further enhancements in the signal were obtained at lower potential values, an electrodeposition potential of-200 mV was selected as optimum. Since the largest response was obtained when t_{edep} = 60 s and E_{edep} = −200 mV were used, these values were selected as optimum and used for all remaining procedures.

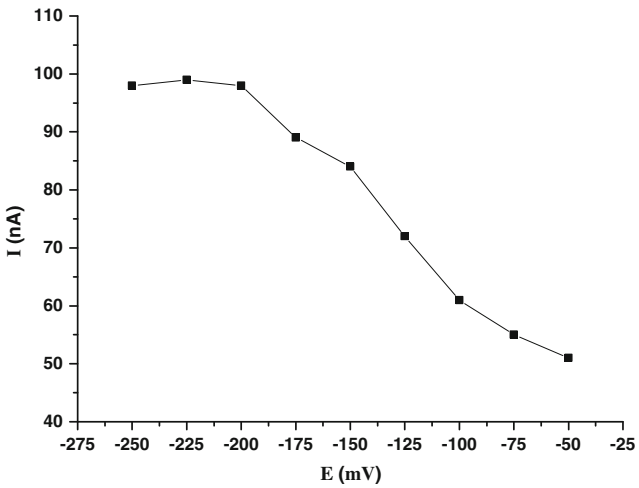


Fig. 3 Study of electrodeposition potential from −50 to −250 mV

Parameter optimization

The following parameters were optimized: (a) Concentrations of H_2O_2; (b) Concentrations of 4-tert-butylcatechol; (c) pH range (Fig. S1); and (d) Enzyme conjugate concentration (Fig. S2). Respective data and figures are given in the Electronic Supporting Material. The following experimental conditions were found to give best results: (a) A H_2O_2 concentration of 1 mmol L^{-1}; (b) A 4-tert-butylcatechol concentration of 1 mmol L^{-1}; (c) A pH of 5.05; (d) An enzyme conjugate concentration of 1:2000.

Table 2 Intra- and inter-day precision and accuracy. Within-assay precision (five measurements in the same run for each control serum, measured as the activity of the correspondent antiserum's enzyme conjugated) and between-assay precision (five measurements for each control serum, repeated for three consecutive days)

	Within-assay		Between-assay	
Reference sera [a]/ng mL^{-1}	Mean	CV%	Mean	CV%
10	9.89	2.95	9.75	3.82
100	100.10	3.98	101.15	5.12
200	200.62	3.13	201.18	4.10

[a] IgM specific antibodies to *T. cruzi*

Table 3 Comparison of the proposed method with previously reported methods for Chagas disease

Detection	Method	Technique	Linear range	LOD	Reference
IgG anti-*T. cruzi*	Microfluidic immunosensor	Amperometric	11–205 ng mL^{-1}	3.065 ng mL^{-1}	[33]
IgG anti-*T. cruzi*	Biosensor	Amperometric	0.031–3.1 μg mL^{-1}	62 ng mL^{-1}	[34]
IgM anti-*T. cruzi*	ELISA	Spectrophotometric	-	-	[24]
IgM anti-*T. cruzi*	Electrochemical immunosensor	Amperometric	10–200 ng mL^{-1}	3.03 ng mL^{-1}	This work

Analytical parameters for the anti-*T. cruzi* specific IgM antibodies detection

The quantification of IgM anti-*T.cruzi* antibodies was evaluated in 20 serum human samples. An anti-*T.cruzi* IgM antibodies calibration plot was obtained by plotting *i* versus anti-*T. cruzi* IgM antibody concentration. A linear relation, ΔI (nA) 43.99 + 0.581 C IgM antibodies, was observed between the *i* and the IgM concentrations in the range from 10 to 200 ng mL^{-1}. The correlation coefficient (r) for this plot was 0.998, where ΔI is the difference between current of the blank and sample (Fig. 4).

The standard deviation (SD) for the calibration curve was 3.5 %. Antibody quantifications for performing the calibration curve were directly carried out on the reference serum (obtained by the procedure by Sanchez-Sus et al. [32]) and its dilutions, due to a matrix effect study carried out showing that it has no influence on the quantification of IgM anti-*T. cruzi* antibodies.

These values demonstrate that our electrochemical immunosensor can be used to quantify the amount of anti-*T. cruzi* IgM-specific antibodies in unknown samples. The detection limit (DL) was considered to be the concentration that gives a signal three times the standard deviation (SD) of the blank. For the electrochemical detection procedure, the DL was 3.03 ng mL^{-1}.

The precision of the electrochemical assay was checked with the control serum at 10, 100, and 200 ng mL^{-1} anti-*T. cruzi* specific antibody concentrations. The within-assay precision was tested with five measurements in the same run for each serum. These series of analyses were repeated for three consecutive days in order to estimate the between-assay precision. The anti-*T.cruzi* assay showed good precision; the CV within-assay values were 4 % and the between-assay values were 5 % (Table 2).

The reproducibility of the electrochemical immunosensor was evaluated with 100 ng mL^{-1} anti-*T. cruzi* antibody. For this a series of six SAPA–MPA–AuNP/SPCE were prepared in the same way and evaluated employing the same conditions described above showing a relative standard deviation (R.S.D.) value of 5.8 % (for $n = 5$).

Regarding the total assay time for the determination of the anti-*T. cruzi* IgM antibody concentration, for this method, the assay time was 26 min, much less than the 90 min normally used with conventional batch well ELISA.

Various methods for quantification of anti- *T. cruzi* specific antibodies have been reported in the scientific literature, including, among others, employ semi-purified antigens from the epimastigote form of *T. cruzi*. Antigens from infectious trypomastigote forms or a combination of *T. cruzi* recombinant proteins and/or synthetic peptides. Table 3 shows a comparison among them.

Conclusions

Our group had previously developed a biosensor for the Chagas disease diagnostic [34], but this electrochemical immunosensor has many differences, one of these is that this method is based on the use of recombinant antigens for the serological diagnosis of *T. cruzi* infection, this has been a clear advance in terms of specificity. Moreover, SAPA reacts with fetal IgM antibodies present in the cord blood from T. cruzi infected newborns. It has been suggested to distinguish congenitally infected infants from uninfected infants by detecting IgM with SAPA. This may allow for unequivocal diagnosis of congenitally infected children at three months of age.

Regarding the results, it is important to note that those obtained using traditional methods are qualitative, while the new device allows the quantitative detection of IgM antibodies. Finally, our device offers the possibility of obtaining miniaturized, integrated and portable systems for on-site analysis.

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Compliance with ethical standard The authors declare that they have no competing interests

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