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Amperometric bioelectrode for specific human immunoglobulin G determination: Optimization of the method to diagnose American trypanosomiasis

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Abstract

Bioelectrodes to detect immunoglobulin G (IgG) antibodies occurring in sera of patients suffering from American trypanosomiasis were assembled. The device consisted of a gold electrode modified with a thiol sensitized with parasite proteins. The assemblage was accomplished by adsorbing IgG antibodies from confirmed infected patients followed by adsorption of anti-human IgG labeled with a redox enzyme. The appliance was used as a working electrode in a three-electrode cell containing a soluble charge-transfer mediator, also behaving as enzyme cosubstrate. The method is based on the measurement of the catalytic current after addition of the enzyme substrate, occurring when a positive serum is used to build up the biosensor. The discrimination efficiency between positive and negative sera was 100% for the samples studied. A 0.9525 correlation coefficient was obtained for results acquired by using this approach and one commercial diagnostic kit. The reproducibility, evaluated by the percentage coefficient of variation, varied between 7 and 20%. The sensitivity was 12.4 ng mL^{-1} IgG, which is in the same order as that obtained with the commercial kit. Stability of the device was studied for a 7-day period and the results showed no significant change ($p = 0.218$). Leishmaniasic sera showed cross-reactivity when total parasite homogenate was used as antigen.
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Immunochemical analysis is applied as a powerful diagnostic tool. Many infectious diseases are routinely diagnosed when specific immunoglobulin G (IgG)[1] antibodies (Abs) against particular antigens (Ags) are detected in the patient's blood by using immunological assays. One of the most frequently used is enzyme-linked immunosorbent assay (ELISA) [1]. ELISA specificity relies on the selective molecular recognition of the Abs generated by the infected patients toward specific Ags, appropriately immobilized on an inert support, commonly sensitized polystyrene microplates. The assay is followed by a second reaction where the specific Ab reacts further with another Ab conjugated with an enzyme label, used to reveal up to which extent the first immunological reaction took place. In this case, either the increase in the amount of one product or the decrease in one of the reactants of the enzymatic reaction is measured, usually by color developing followed by

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[1] *Abbreviations used:* IgG, human immunoglobulin G; Abs, antibodies; Ags, antigens; ELISA, enzyme-linked immunosorbent assay; IHA, indirect hemagglutination; IIF, indirect immunofluorescence; MPA, 3-mercapto-1-propionic acid; MPSA, 3-mercapto-1-propanesulfonic acid; FcMe, ferrocenemethanol; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; HRP-conjugated IgG, HRP-labeled goat anti-human IgG gamma chain; carbodiimide, 1,3-diisopropylcarbodiimide; SD, standard deviation; ANOVA, analysis of variance; CV (%), percentage coefficient of variation; *R*, correlation coefficient; 2-D, two dimensional.

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spectrophotometric detection. These techniques require relatively expensive kits, which utilize disposable microplates sensitized with the antigen. The development of more sensitive, faster, easier-to-use, and lower-cost methods is therefore of crucial importance for public health, since it can help to diagnose the disease at a social scale.

Biosensors offer promising means to achieve this goal, particularly due to the relatively simple and inexpensive equipment required, their high sensitivity, and their potential application for automatization [2–4]. Diverse immunosensors have been described, mostly aimed to detect Ags, for which direct or competitive immunoassay formats have been developed [5–7].

Anti-*Trypanosoma cruzi* IgGs are the expected Abs to be found in chronic patients suffering from American trypanosomiasis, also known as Chagas–Mazza's disease. Nowadays, this pathology is considered to be the most serious American parasitosis, since about 20 million people are infected in Latin America alone [8]. Although it would be desirable to detect the infection during the acute phase occurring with patient parasitemia, in most cases this is not possible because of the short duration of this phase and the low parasite concentration in blood. Therefore, the diagnosis of this parasitosis is currently achieved during the chronic phase, when *T. cruzi* Abs are detected in the patient's serum. However, serologic methods have the potential drawback of cross-reactions with related protozoans, particularly Leishmania. To enhance the sensitivity and specificity of serologic diagnosis, at least two independent tests must be conducted [9]. Even when using two different methods, there are inconclusive results ranging from 2 to 19%, depending on the combination of methods considered [10]. The most widely used assays for this purpose are indirect hemagglutination (IHA), indirect immunofluorescence (IIF), and ELISA [9].

An impedimetric evaluation of electrode/*T. cruzi*-antigen interface has been performed by exposing these electrodes to positive and negative chagasic sera, suggesting the potential use of this methodology for the diagnosis of the parasitosis [11]. With this methodology, however, reproducibility problems were reported. To the best of our knowledge, no further studies on the assessment of impedimetric methodology with regard to improved sensitivity or selectivity, as compared with those of the currently used ELISA, IIF, or IHA methods, have been reported.

The interest in developing a new immunological assay arises from its potential improvement when combined with electrochemical detection. To the high selectivity and specificity of the antigen–Ab interaction, an added value is obtained when the assay sensitivity is enhanced using amperometric detection. Moreover, counting with a portable chagasic biosensor technology will offer the possibility of testing patients' infection in the field. This is of crucial importance in many developing countries, where personnel dealing with primary health attention regularly visit people suffering from this disease who live far from hospitals counting with microplate ELISA readers.

Diverse amperometric biosensors have been proposed to detect a vast number of analytes of interest in very different areas, such as agriculture, medicine, toxicology, food production, and environmental chemistry, among others [6,11–16]. One advantage of using amperometric biosensors instead of classical methods is the ability to operate in turbid, complex or colored matrices. This reduces considerably, or even prevents, sample pretreatment steps, allowing the analysis to be faster and less expensive. Moreover, by detecting electroactive substrates or products of one enzymatic reaction, it is possible to improve sensitivity, since an effective amplification of the measured signal occurs [17,18].

In this work, we describe a new device to detect the presence of specific, chagasic-IgG Abs in human sera, consisting of an anti-*T. cruzi* IgG Ab biosensor built up on a metallic electrode, with which an indirect ELISA is performed. The electrode performances obtained under different working conditions during assemblage were compared to establish the best strategy to build up the device. Finally, the method was compared with results obtained with one ELISA commercial kit.

Experimental

Chemicals and reagents

Hydrogen peroxide was supplied by Riedel-de Haën. Thiols, 3-mercapto-1-propionic acid (MPA), 3-mercapto-1-propanesulfonic acid (MPSA), 1,3-diisopropylcarbodiimide (carbodiimide), and Tween 20 were from Aldrich. Sulfuric acid, salts to prepare phosphate-buffered saline (PBS; 0.16 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, and 0.0018 M KH_2PO_4), ferrocenemethanol (FcMe), phenol, 4-aminoantipyrine, casein sodium salt from bovine milk, sodium chloride, and potassium chloride were obtained from Sigma. All chemicals were of analytical grade.

Horseradish peroxidase (HRP)-labeled goat anti-human IgG gamma chain (HRP-conjugated IgG) was from Zymed.

Obtainment of the antigen

Trypanosoma cruzi epimastigotes were harvested, washed in PBS, and resuspended in 5 volumes of 1 mM tosyl-L-lysine chloro-methyl ketone and 1 mM phenylmethylsulfonyl fluoride in distilled water. After being frozen and thawed four times, the suspension was sonicated (20 kHz, 30 W, 2 min). The lysate containing the parasite antigen was cleared by centrifugation at 15,000*g* for 15 min, and the supernatant was aliquoted and kept at −20 °C until use. Immediately prior to use, the parasite lysate was defrosted and then sonicated for 3 min. Total protein determination was carried out by the Lowry's method [19].

Characterization of the parasite homogenate

Antigenic proteins of the parasite were characterized by two-dimensional (2-D) electrophoresis, using the procedure described by O'Farrell [20], with pertinent modifications to be used in a mini 2-D electrophoresis cell (Bio-Rad). Isoelectrofocusing of antigenic proteins was performed at the first dimension of the electrophoresis and SDS–PAGE at the second dimension. Parasite proteins were transferred to a nitrocellulose membrane, treated with a pool of sera positive for American trypanosomiasis, and incubated with HRP-conjugated IgG. The procedure I.U.B.: 1.11.1.7, Worthington catalogue (see below, Spectrophotometric experiments), was used to reveal the system. Molecular masses of the antigen ranged between 20 and 90 kDa, the bulk belonging to the 40- to 50-kDa range. The isoelectric point ranged from 5.9 to 6.3.

Patients' sera

Sera obtained from confirmed, chronic *T. cruzi*-infected patients were tested to be positive by using two immunological methods, namely, ELISA with spectrophotometric detection and IHA. Negative sera were obtained from individuals whose serum sample rendered negative results by using both methods.

Aliquots of positive sera were pooled, and dilutions of this pool were used to obtain the calibration curve. The pool titer was determined by the following procedure: 25.0 mg of parasite homogenate proteins was fixed in a 3.5-mL column of BrCN-agarose (Amersham). The serum pool (0.5 mL diluted in 10 mL of PBS) was passed five times through the column for the specific Abs to be retained. This was confirmed by performing ELISA on the flow through, where no specific Ab was detected. The column was then rinsed with 10 column volumes of PBS, specific Abs not having been detected by ELISA in the flow-through buffer. The Abs previously adsorbed were eluted with 0.1 M glycine HCl buffer, pH 2.8. The fractions recovered in the first 4 column volumes of eluate were those containing the specific Abs, as evaluated by ELISA. Recovered Abs were concentrated in 20-to-1 volumes, and their concentrations were determined by Bradford's method [21]. Ab concentration was 31.0 $\mu g\ mL^{-1}$ of IgG.

Electrode preparation

Gold electrodes of average geometric area ca. 0.6 cm^2 were cleaned deeply with piranha solution, 18 M H_2SO_4:30 g/100 mL H_2O_2 (3:1), thoroughly rinsed with distilled water, and sonicated for 5 min in bidistilled water.[2] The cleanness of the electrode surface was then checked by cyclic voltammetry in 2 M H_2SO_4 solution, at 0.050 $V\ s^{-1}$, from 0 to 1.700 V vs Ag/AgCl reference electrode.

Electrodes were modified immediately after the cleaning step. Thiol solutions were freshly prepared in distilled water, previously bubbled with nitrogen. Initial thiol adsorption was accomplished by immersing the electrodes for 12 h in either 10 mM MPSA solution in 10 mM H_2SO_4 or 10 mM MPA solution in water, depending on the experiment. After thiol adsorption, the electrodes were thoroughly rinsed with distilled water and sonicated for 2 min.

The following assemblage step depended on the strategy used to bind the antigen to the electrode. When electrostatic binding was assayed, the *T. cruzi* antigen was deposited directly onto the thiol-modified surface by soaking the electrodes in PBS containing the parasite antigen, for 2 h at 37 °C. The same procedure was followed either at pH 7.4 or at pH 5.0 to ascertain performance differences under these two conditions and reveal the predominant nature of the binding (electrostatic or hydrophobic). Alternatively, the antigen was bound covalently to the electrodes. For this purpose, the electrodes were treated with 10 g/100 mL carbodiimide in 0.01 M phosphate buffer, pH 7, for 2 h, followed by soaking of the electrodes in the PBS-diluted antigen. The electrodes were further rinsed thrice with PBS and then submerged in 10 $mg\ mL^{-1}$ bovine casein sodium salt in PBS, for 10 min at 37 °C to hinder the unbound thiolated sites [22]. The *T. cruzi*-sensitized electrodes thus prepared were stored at 4 °C in the casein solution until performing the indirect immunoassay.

Indirect immunoassay

Immediately prior to use, the electrodes were rinsed thrice with 0.05 g/100 mL Tween 20 in PBS (PBS-T) and incubated for 30 min at 37 °C, in the human serum sample to be tested, commonly (1:200) diluted in PBS. However, to carry out the calibration curve, the positive-serum pool was diluted in PBS in the range 1:20 to 1:10,000. After carefully washing the electrodes with PBS, the electrodes were incubated for 30 min at 37 °C, either in HRP-conjugated IgG at several dilutions in PBS (to determine the best condition) or in 0.07 mL/100 mL HRP-conjugated IgG solution in PBS (the conjugate concentration that rendered optimum results). After the assemblage was finished, the electrodes were washed thoroughly with PBS and kept in the same buffer until performing either the spectrophotometric or the electrochemical experiments, usually within the following hour, except when the device stability was assayed, in which case the longest storage period was 7 days.

Spectrophotometric experiments

Electrodes were submerged for 6 min into a plastic semimicro cuvette containing 0.7 mL of the enzyme substrate solution (1.8 mM H_2O_2) and the hydrogen donor

[2] We used gold-flag electrodes as a prototypical model to set up the methodology, since this allowed us to reuse the electrodes by regenerating their surface using piranha solution. However, smaller commercial gold electrodes, for example gold screen-printed electrodes, could also be used.

(1.25 mM 4-aminoantipyrine with 85 mM phenol) in PBS at room temperature (22–27 °C). The assay is based on the procedure described in the Worthington catalogue, I.U.B.: 1.11.1.7., which uses 4-aminoantipyrine as hydrogen donor and the above-stated concentrations of the reactants [23]. After the incubation period, electrodes were rinsed and kept in PBS for further electrochemical experiments. Absorbance was measured at 510 nm with a Beckman DU 640 spectrophotometer, after 15 min of color stabilization.

Electrochemical experiments

An Autolab Electrochemical Analyzer with PGSTAT 30 software was used for all electrochemical experiments. A large-surfaced, gold electrode and an Ag/AgCl electrode were used as counter and reference electrodes, respectively, as part of a conventional three-electrode cell. All potentials were measured and therefore quoted with respect to Ag/AgCl.

The electrode response was assessed by amperometry, at a constant potential (0.100 V vs Ag/AgCl) and either at 25 °C or at room temperature (22–27 °C), in an attempt to emulate the conditions under which the potential users of the device will be supposed to work routinely. The system was allowed to equilibrate at the working voltage in 3.0 mM FcMe solution in PBS, and the background current was then recorded. A 90 mM H_2O_2 solution was added to reach a final 1.8 mM substrate concentration, i.e., the same concentration as that recommended in the Worthington catalogue (see above). The current was then monitored and measured after stabilization.

Since we used home made gold-flag electrodes, we normalized the signal with respect to the area. However, this procedure can be omitted when using standardized commercial electrodes, which are expected to have comparable areas. Current–potential waves of FcMe in PBS were acquired for each electrode at different voltage sweep rates. From these curves, the current peaks, i_p, were obtained. The slope of the regression line in a plot i_p vs square root of the voltage sweep rate allowed obtaining the electrode area, by using the Randles–Sevcik equation [24].

Statistical analysis

Results were expressed as mean ± SD. A Student's t test was used for comparisons, with previous verification of the normal variance distribution. One-way ANOVA was used for multiple comparisons. The level of significance was set at $p < 0.05$. The percentage coefficient of variation, CV (%), calculated as SD × 100/mean, was used to evaluate reproducibility of results.

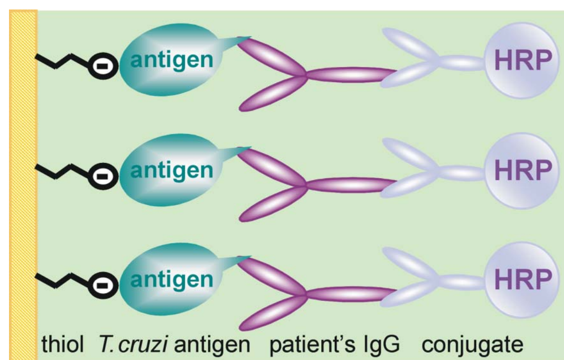
Results and discussion

Several articles describing competitive immunoassays with electrochemical detection to detect a wide range of Ags have been published [6,11,25]. In addition, a direct, enzyme-amplified, amperometric assay for affinity systems, such as avidin–biotin conjugate, has been reported [26]. In this work, we propose as a new strategy an indirect immunoassay with amperometric detection to assess anti-*T. cruzi* Abs. An indirect ELISA system rather than a competitive system was preferred for IgG detection since (i) it is extremely difficult to set up a competitive assay to assess the presence of Abs because the avidity of these Abs toward the antigen is very variable among infected individuals, and the same holds true for Abs concentration, which may vary within a very wide range (~5 orders of magnitude), depending on the individual extent of the immunological response [27]; (ii) indirect ELISA utilizes secondary, enzyme-labeled IgG toward human IgG, which are readily available from commercial sources; in contrast, competitive ELISA utilizes specific monoclonal IgG human Abs labeled with the redox enzyme, whose synthesis requires costly and relatively high technology to be applied; and (iii) currently available diagnostic kits are based upon indirect ELISA systems with spectrophotometric detection, so that our approach with regard to sensitivity can be directly compared with them [28].

General outline of the method

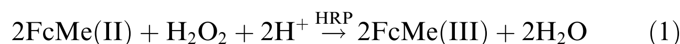
We explored the possibility of developing a classical indirect ELISA aimed to determine specific chagasic-IgG Abs, replacing the spectrophotometric detection with electrochemical detection. For that reason, not only the sensitized element but also the detection procedure was changed. Instead of developing a colored product via the enzymatic reaction, we incorporated in the last step a soluble charge mediator to detect by amperometry the presence of the enzyme label.

To achieve this goal, we assembled a bioreactive layer on a modified gold surface, which contains the analyte (specific anti-*T. cruzi* IgG) and an enzyme label, HRP. Scheme 1 depicts a graphic representation of this system, which was used as working electrode in a conventional

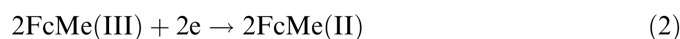


Scheme 1. Schematic representation of the biosensing device, gold electrode/thiol/*Trypanosoma cruzi* antigen/patient's antibody/horseradish peroxidase-conjugated anti-human IgG.

three-electrode cell to perform an amperometric experiment in presence of the substrate of the HRP, H_2O_2, and a soluble mediator, FcMe, working as a cosubstrate of the enzyme label. Under these conditions, HRP oxidizes the cosubstrate, which will then mediate the charge transport between the heme prosthetic group of the enzyme and the electrode, FcMe being reduced at the electrode surface at an appropriate potential. The catalytic cycle is described by the following equations:



$$2\text{FcMe(II)} + H_2O_2 + 2H^+ \xrightarrow{\text{HRP}} 2\text{FcMe(III)} + 2H_2O \quad (1)$$



$$2\text{FcMe(III)} + 2e \rightarrow 2\text{FcMe(II)} \quad (2)$$

The detection of a reduction current signal, in consequence, evidences the occurrence of the catalytic cycle, demonstrating the presence of the analyte in the sample; the higher the titer of anti-*T. cruzi* Abs in the sample, the higher the expected current to be measured.

The cosubstrate, FcMe, is responsible for the mediation between the redox site of the enzyme and the gold surface. This mediation is easily monitored amperometrically by measuring the cathodic current increase when the enzyme substrate, H_2O_2, is added, thus evidencing the connection between the conjugate and the electrode surface.

Fig. 1 shows a three-run, cyclic voltammogram of 3.0 mM FcMe solution in PBS using the device proposed, registered at 0.05 V s^{-1} voltage sweep rate, starting potential −0.06 V, and final potential 0.55 V. From Fig. 1 it can be inferred that the charge mediator FcMe diffuses through the bioreactive layer. The inset in Fig. 1 depicts the steady state catalytic response of an electrode built up using a (1:200) dilution of a Chagas-positive serum, in a 3.0 mM FcMe solution in PBS containing 1.8 mM H_2O_2, recorded at several voltages, and corrected by deducting the background current measured before adding the enzyme substrate. By examining the Fig. 1 inset, it is apparent that, under these working conditions, the cathodic current remains constant from 0.100 V to lower potentials. We therefore performed the amperometry by recording the cathodic current at a working electrode potential of 0.100 V vs the reference electrode.

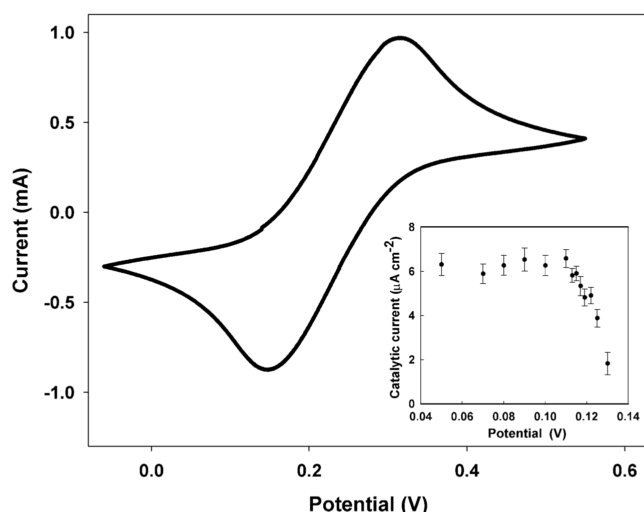


Fig. 1. Cyclic voltammetry of the charge transfer mediator, FcMe. Cyclic voltammogram of 3.0 mM FcMe solution in PBS using one electrode assembled with a positive serum sample and registered at 0.05 V s^{-1} voltage sweep rate, starting potential −0.06 V, and final potential 0.55 V. Inset: catalytic current vs applied potential. Steady state current–voltage curve obtained for an electrode assembled with one positive sample (1:200) diluted in 3.0 mM FcMe with 1.8 mM H_2O_2 in PBS, corrected for the background current obtained before enzyme substrate addition.

Electrode assembling process

To build up the electrodes, we used the self-assembly technique. Particularly, we focused on obtaining the deposit of an ordered layer, which is achieved by immersing the electrodes consecutively into the following solutions: (a) thiol, to modify the gold to obtain a surface which exposes residues that allow either electrostatic or covalent binding to the antigen, (b) parasite protein Ags, to further bound specific Abs, (c) bovine casein, to hinder unmodified sites, (d) the sample containing the specific IgG Abs, and (e) anti-human IgG conjugated with HRP, which will bound to the analyte attached in the previous step.

Our approach was, in principle, designed to follow a working protocol not longer or more complicated than that followed by an analyst who uses any of the ELISA kits currently available for American trypanosomiasis diagnosis.[3] To minimize differences with this method, we reproduced the same assemblage sequence and assay conditions used in the Chagatest kit (Wiener Lab, Argentina), specifically at those common steps in which the same reagents are used. Consequently, incubation periods to allow antigen–Ab reactions were the same as those used in the commercial kit. Ancillary experiments were carried out to determine both the optimal period of exposure time to thiols of the clean gold surface and the optimal concentrations of thiols and HRP-conjugated IgG.

To determine whether the assembled electrodes showed enzymatic activity, spectrophotometric determinations were performed. These experiments allow verifying any mediation problem since, if that occurs, enzymatic activity is registered spectrophotometrically, but no concomitant reduction current should be registered when performing the amperometry.

It has to be considered that the signal measured, whether by a spectroscopic or an electrochemical method, depends on the amount of enzyme-conjugated IgG bound to the electrode, which in turn depends on the area of the electrode. As we used different gold flags to build up the

[3] The "ready-to-use electrodes" with which the analyst is expected to work should be considered devices alternative to the sensitized microplates of commercial ELISA kits. Therefore, the total time to perform the analysis must be considered from the serum sample incubation steps onward. All previous assemblage steps are not supposed to be followed by the potential user of this device, in the same way that he/she does not sensitize ELISA kit microplates.

electrodes, all data (either of absorbance or of catalytic current) were corrected by dividing them by the electrode area.

Binding strategy selection

To test different ways to adsorb the antigen, we first modified the clean gold surface with two different thiols, MPSA and MPA, which provide sulfonate or carboxylic residues, respectively, for the electrostatic binding of the antigen. Furthermore, to provide evidences that electrostatic interactions are involved, we worked under different pH conditions, since the charge of the antigen changes with the degree of protein protonation, thus influencing electrostatic forces between the antigen and the sulfonate or carboxylic residue modifying the gold surface. For this purpose, we determined first the p*I* range of the antigen homogenate used in these experiments by isoelectrofocusing, which turned out to be 5.9–6.2. Therefore, when working at pH 5.0, most of the antigen should be positively charged, whereas, at this pH, the sulfonate or carboxylic residues of MPSA or MPA, respectively, should be negatively charged. The reduction currents obtained for six replicates of this experiment were 4.2 ± 0.3 and $3.9 \pm 0.3\ \mu A\ cm^{-2}$ for MPSA and MPA, respectively, having used one positive serum sample (1:200) diluted in PBS to assemble the electrode. When working at pH 7.4, a condition under which both the antigen and the sulfonate or carboxylic residues are expected to be mainly negatively charged, the antigen binding was less effective, as shown by the lower enzymatic activity measured by both spectrophotometry and amperometry. At this pH, the reduction currents for MPSA and MPA were 3.5 ± 0.2 and $3.3 \pm 0.2\ \mu A\ cm^{-2}$, respectively ($p < 0.05$ vs pH 5.0), having used the same positive serum sample (1:200) diluted in PBS. We therefore conclude that electrostatic forces are involved in the adsorption of the antigen to the thiolated gold surface, in accordance with previous studies [29,30]. However, other attractive forces such as hydrogen-bond or hydrophobic forces cannot be ruled out to contribute in the assembling process also as suggested by others [31].

Since we build single-layered systems and, therefore, there is little material immobilized on the electrode's surface, it seems reasonable that diminutions of the antigenic protein due to desorption during the assembling process lead to lower final enzymatic activities [32–34]. To prevent antigen desorption, we attempted to strengthen the attachment of the antigen to the modified gold surface by activating the terminal group of the thiol with carbodiimide. This compound allows covalent linkage of either the terminal–SO_3H (for MPSA) or the –COOH (of MPA) with the terminal amine residues of the antigen structure [1,22,35]. One would therefore expect that covalent binding of the antigen enhances the electrode performance by increasing the signal-to-noise ratio. In conflict with our original expectation, we observed no significant improvement of the bioelectrode performance when doing so. Indeed, the catalytic currents obtained with electrodes built up by favoring electrostatic interactions were 4.2 ± 0.3 and $3.9 \pm 0.3\ \mu A\ cm^{-2}$ for MPSA and MPA, respectively, whereas the currents measured for electrodes with the antigen covalently bound to the thiolated surface were 3.3 ± 0.3 and $3.6 \pm 0.3\ \mu A\ cm^{-2}$, respectively. Statistical analysis (*t* test) showed no difference between groups ($p > 0.05$). An hypothesis that should still be investigated to explain our results is that the only covalent bonds between the electrode and the antigen that are effective, with regard to signal, are those allowing appropriate orientation of the protein for subsequent recognition of the specific Ab. Any substitution leading to hindrance or even blockage of those terminal amine residues involved in the antigen–Ab reaction will diminish the effectiveness of the assemblage process. If this is true, only a fraction of the antigenic proteins present will be linked with the convenient orientation to allow subsequent recognition by the specific Abs. On the contrary, when the antigen is adsorbed to the thiolated surface via electrostatic interactions, even when the antigen is not conveniently orientated, the strong interactions between the antigen and its high-affinity, specific Abs can induce a redirection of the protein, leading to a higher amount of conveniently orientated analyte.

Spectroscopic and amperometric methods were used to compare the effectiveness of the assemblage process with both MPSA and MPA by favoring either electrostatic or covalent unions. Table 1 shows a synopsis of the catalytic currents measured using electrodes built up under each of the mentioned conditions. Comparison of the results for all the assembling strategies (six replicates each) indicates that the best results are obtained when using MPSA at pH 5 to favor electrostatic interactions. Since no significant improvement of the electrode performance was observed by covalently binding the antigen, in further experiments we attached the antigen proteins using MPSA, at pH 5.0.

Concentration of T. cruzi antigen

Like most ELISAs, the optimal dilution of the antigen had to be determined in our system. Therefore, the same protocol was repeated for different antigen concentrations,

Table 1
Catalytic current recorded with electrodes assembled under several different conditions, using the same positive serum sample (1:200) diluted in PBS[a], and six replicate electrodes under each condition

Thiol	pH of Ag [b] solution	Electrode response/$\mu A\ cm^{-2}$	
		Noncovalent Ag binding	Covalent Ag binding
MPSA[c]	5	4.2 ± 0.3	—
	7.4	3.5 ± 0.2	3.3 ± 0.3
MPA[d]	5	3.9 ± 0.3	—
	7.4	3.3 ± 0.2	3.6 ± 0.3

[a] PBS, phosphate-buffered saline.
[b] Ag, antigen.
[c] MPSA, 3-mercapto-1-propanesulfonic acid.
[d] MPA, 3-mercapto-1-propionic acid.

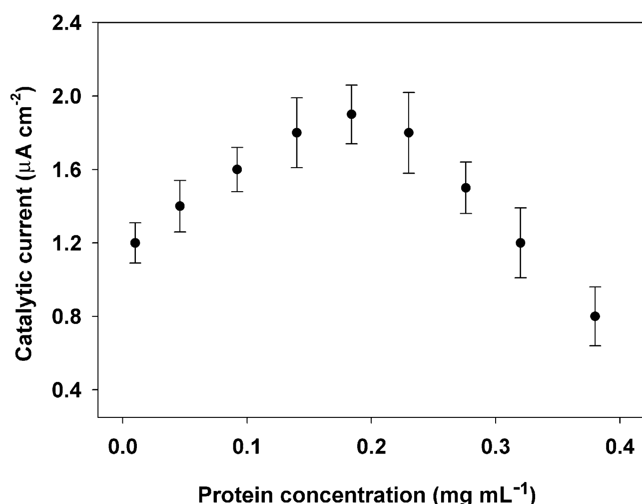


Fig. 2. Signal obtained vs protein concentration used during the electrode assemblage. Catalytic current as a function of *Trypanosoma cruzi* protein total concentration, measured with electrodes assembled using the same positive serum sample (1:200) diluted in PBS.

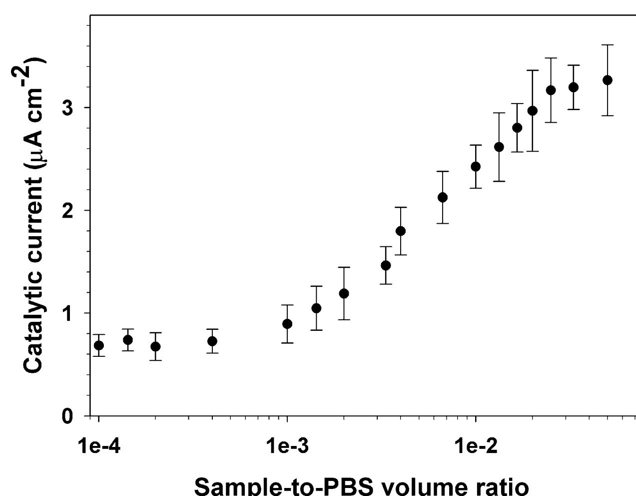


Fig. 3. Signal obtained vs antibody concentration used during the electrode assemblage. Catalytic current recorded at 0.100 V constant voltage vs Ag/AgCl, in 3.0 mM FcMe, with 1.8 mM H_2O_2 in PBS, for series of five electrodes each, assembled with decreasing dilutions of Chagas' IgG antibodies (expressed as sample-to-PBS volume ratio). The catalytic current was corrected by the background current measured before addition of the enzyme substrate.

recording the amperometries at 25 °C to find the best binding isotherm. Results obtained with electrodes built up using different concentrations of the same *T. cruzi* homogenate, the same positive serum sample dilution (1:200), and the same anti-IgG-conjugate solution are shown in Fig. 2. It can be seen that, for MPSA-modified electrodes, the signal increases with total protein homogenate concentration, probably due to the increasing coating of the modified sites of the electrode by the antigen. However, the signal achieves a maximum and, from that point onward it diminishes. This behavior can be explained by the fact that working at relatively high antigen concentrations may lead to multiple protein layer formation [36]. Taking into account that some of the noncovalently adsorbed Ags may be desorbed during testing [36], the released parasite protein can therefore compete with the adsorbed protein for the Ab present in the sample, eventually decreasing the signal detected. Our results indicate that the optimal antigen concentration is 0.18 mg mL^{-1}, expressed in total protein concentration, for MPSA-modified electrodes.

Reproducibility of the method

To measure reproducibility of results using this immunosensing device, amperometric experiments were performed at 25 °C for electrodes prepared using different dilutions of the same pooled positive sera containing 31.0 μg mL^{-1} specific IgG. A calibration curve of the catalytic current, measured as a function of Ab concentration, is shown in Fig. 3. Seventeen different pool dilutions were analyzed (using five different electrodes for each dilution). The reproducibility of the method was evaluated by assessing CV (%) at each dilution. The values obtained were within the 7–20% range. Commercial ELISA kits report as an acceptable interassay variability CV (%) values lower than 20%. Our results therefore indicate that our method fits the accepted interassay variability criterion for currently used ELISA diagnostic kits.

Assay specificity and cutoff value

Sera of infected human patients contain many other proteins in addition to specific anti-*T. cruzi* Abs, which might lead to cross-reactivity. Consequently, the presence of such compounds in natural samples may produce interference and should be evaluated. We therefore assembled electrodes with negative samples, expecting to record negligible catalytic currents. Reduction currents ranging from 5 to 15% of those measured for the positive-serum pool were recorded for the negative samples tested. Although in other works the electrodes are rinsed with saline buffer solutions throughout the assembling process [22,29], we assayed PBS-T as washing solution in every step where an antigen–Ab reaction is expected to take place to prevent nonspecific reactions. However, the possibility that Tween 20 reduces also the extent of the desired reaction cannot be ruled out. Ancillary experiments comparing results with both washing solutions showed that PBS-T washing diminished only by 5% the average signal for positive samples. However, Chagas-negative sera also rendered lower signals, suggesting that there is less unspecific attachment when using PBS-T, as compared with PBS alone, in line with theory [27].

Amperometric tests performed with electrodes assembled with a (1:20) dilution[4] of each negative serum sample

[4] The sera dilution used in these experiments (1:20) is lower than those used in most of the work because, under these conditions, any cross-reactivity producing signal will be enhanced. Therefore, the cut-off value estimated under this maximally unfavorable condition allowed obtaining reliable positive results.

($n = 11$) rendered a current of $0.42 \pm 0.06\ \mu A\ cm^{-2}$, as compared with $3.3 \pm 0.3\ \mu A\ cm^{-2}$ obtained for the same dilution of the positive pooled sera. As can be seen, the electrodes assembled with negative sera still produce some signal, even when using PBS-T as washing solution. Though cross-reactions are apparent, the average result determined for negative samples represents only 13% of the signal obtained with the same dilution of the positive pooled samples. We calculated the cutoff value for this immunoassay with amperometric detection as thrice the standard deviation added to the average current obtained for the negative sera panel studied [27]. According to this criterion, we consider a sample to be positive for American trypanosomiasis when the current measured at the present working conditions is over $0.60\ \mu A\ cm^{-2}$.

Method comparison

To assess the validity of the proposed method, it is essential to demonstrate that the results obtained with our approach agree with those obtained using other methods. For this purpose, assays were performed using electrodes assembled with 14 different (1:100) diluted serum samples, which had been previously typified as positive or negative for American trypanosomiasis by means of ELISA and IHA. Fig. 4 depicts the signals measured by using the new approach as a function of those obtained by classical ELISA for the 14 tested samples. The correlation coefficient, R, was 0.9525, a value that indicates that there is an acceptable correlation between both methods. It has to be pointed out that all positive samples ($n = 7$) rendered currents above the cutoff value, $0.60\ \mu A\ cm^{-2}$, whereas all negative samples ($n = 7$) rendered values lower than $0.60\ \mu A\ cm^{-2}$. Therefore, the discrimination efficiency of the amperometric approach was the same as those of ELISA and IHA for the samples studied.

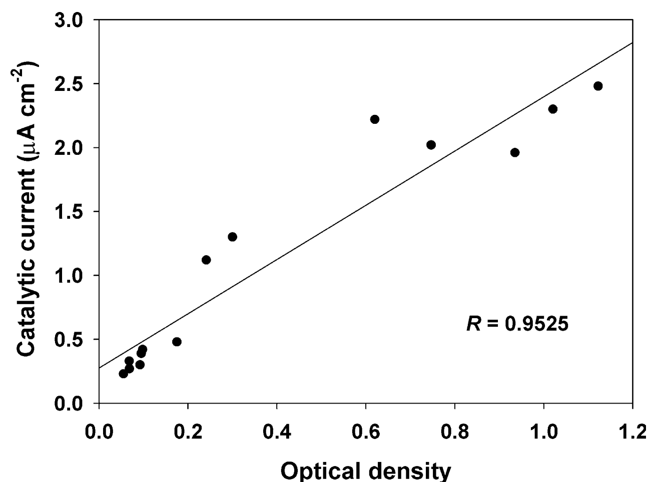


Fig. 4. Comparison of signals obtained using the proposed device vs those obtained by classical ELISA. Catalytic currents measured for seven positive and seven negative serum samples as a function of the optical density obtained by using ELISA with spectrophotometric detection for the same serum samples.

To evaluate the sensitivity of the new approach as compared with that of ELISA with spectrophotometric detection, we determined a positive-serum pool titer by both methods. When using the commercial ELISA kit, a 1/2560 titer was obtained, which corresponds to $12.0\ ng\ mL^{-1}$ of anti-*T. curzi* specific IgG. When using our approach to determine the pool titer, this turned out to be 1/2500, which corresponds to $12.4\ ng\ mL^{-1}$. These results suggest that both methods are equivalent with regard to sensitivity.

To compare the specificity of our approach with that of routine ELISA, we tested two different serum samples negative for American trypanosomiasis but positive for Leishmania. The ELISA commercial kit used for this experiment was one containing total homogenate parasite to keep the same sensitizing antigen as that attached to the electrodes. Both of the methods rendered false positive results. These results indicate that more specificity is clearly required to discriminate both parasitoses. This goal could be achieved by using recombinant proteins as electrode sensitizing agent, aiming to avoid unspecific Ab binding. The good responses obtained for electrodes built up with total homogenate parasite have shown that the proposed strategy is viable, thus encouraging us to probe assembling the biosensor using *T. cruzi* recombinant proteins. Consequently, new experiments using a chimera *T. cruzi* recombinant protein are now in progress, in an attempt to reduce cross-reactivity and improve the performance of the device.

Stability of the device

We studied the stability of the electrode once we accomplished the assemblage. For this purpose, 20 electrodes were built up simultaneously under the same conditions, namely, MPSA as thiol, $0.18\ mg\ mL^{-1}$ total protein homogenate as sensitizing antigen, $31.0\ \mu g\ mL^{-1}$ specific-IgG from serum pool (1:200) diluted in PBS as sample, $10\ mg\ mL^{-1}$ casein solution as blocking agent of unmodified sites, and 0.07 mL/100 mL HRP-conjugated IgG as enzyme label. In an attempt to keep equivalent conditions unchanged, the electrodes were immersed together in one ad hoc built cuvette, which allowed them to be in the same solution but prevented contact with each other. The electrodes thus built up were stored in PBS at 4 °C for a period of time ranging from 0 to 7 days. Amperometric experiments at days 0, 1, 3, 5, and 7 were performed, at 25 °C, for four different electrodes each day. The reduction currents in the sets of four replicate electrodes, measured at the five different periods of time, were similar to each other (5-day mean value: $2.1 \pm 0.4\ \mu A\ cm^{-2}$), as shown by one-way ANOVA test ($p = 0.218$). We therefore concluded that the electrode performance did not vary within the storage period studied.

Experiments are in progress to verify the stability of the ready to use device, as it is supposed to arrive at the analyst

hands, i.e., the electrode sensitized with the antigen and with unmodified sites blocked with casein.

Conclusions

A new biosensor was developed, characterized, and optimized to determine human anti-*T. cruzi* IgG. The immunoassay was performed using FcMe as the soluble mediator, and the detection was carried out by amperometry. The electrochemical studies showed that the best electrode performance was reached when using MPSA as thiol and when favoring attractive electrostatic forces between the antigen and the electrode surface, the reduction current increased with the concentration of specific IgG Abs, the organized layer of the device built up at the optimal studied condition did not limit FcMe diffusion, thus allowing charge transport between the enzyme conjugate and the electrode surface, in agreement with the proposed catalytic cycle, all tested sera, previously verified to be positive for American trypanosomiasis, rendered positive results when using the new approach, and the comparison between the results obtained with the proposed method and those obtained with one currently used commercial ELISA kit demonstrated that anti-*T. cruzi* IgG can be determined successfully by this method, as far as sensitivity and specificity are concerned.

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