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Favorably orienting recombinant proteins to develop amperometric biosensors to diagnose Chagas' disease

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ABSTRACT

Clinical immunoassays often display suitable sensitivity but some lack of specificity or vice versa. As a trade-off between specificity improvement and sensitivity loss, biosensors were designed to perform indirect immunoassays with amperometric detection using tailor-made chimeric receptors to react with the analyte, specific anti-*Trypanosoma cruzi* immunoglobulin G (IgG). Recombinant chimeras were designed to favor their oriented covalent attachment. This allows the chimeras to properly expose their epitopes, to efficiently capture the analyte, and to withstand severe chemical treatment to reuse the biosensors. By further binding the secondary antibody, horseradish peroxidase-labeled anti-human IgG, in the presence of the soluble mediator and the enzyme substrate, a current that increased with the analyte concentration was measured. Biosensors using the chimeric constructions showed 100% specificity with samples that had revealed false-positive results when using other bioreceptors. A protein bearing a poly-Lys chain and thioredoxin as directing elements displayed the highest signal-to-noise ratio ($P < 0.05$). The limit of detection was 62 ng ml^{-1}, which is eight times lower than that obtained with a currently used commercial Chagas enzyme-linked immunosorbent assay (ELISA) kit. Reusability of the biosensor was assessed. The signal was approximately 80% of the original one after performing 10 consecutive determinations.

The development of biosensors with clinical diagnostic purposes has been a matter of increasing interest during recent years due to their notable advantages as compared with classical methods [1–3]. Briefly, these advantages include the ability of working without sample pretreatment, rapid analysis, low cost of instrumentation, and the reliability of the obtained results because of their usual high sensitivity and specificity. Moreover, biosensors offer the possibility of performing assays in the field, and they have the potential to be used in automatic analyzers [4,5].

One challenge of designing a biosensor is preserving the ability of the biological species to capture the analyte by exposing its specific binding sites. A number of attempts have been made to achieve this goal, including keeping glucose oxidase in a layer of highly ordered polyaniline nanotubes [6], immobilizing a consensus double-stranded DNA onto a carboxymethylated dextran film using an aminated oligonucleotide [7], covalently binding *Desulfovibrio gigas* hydrogenase to a functionalized carbon electrode [8], and oriented binding of a specific capturing antibody (Ab)[1] on a pre-cross-linked polymer via protein A [9]. All of these strategies aimed to increase the readily available, biologically active species on the sensor surface so as to render enhanced sensitivity. Here we evaluated an alternative and effective way to favorably direct the analyte-capturing molecule via a chemical reaction between a carbodiimide-activated, gold-modified surface with specifically designed recombinant amino-enriched proteins. Here 3 and 13 extra

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[1] *Abbreviations used:* Ab, antibody; Ang, antigen; ELISA, enzyme-linked immunosorbent assay; IHA, indirect hemagglutination; IIF, indirect immunofluorescence; thiol, 3-mercapto-1-propanesulfonic acid; carbodiimide, 1,3-diisopropylcarbodiimide; T, Tween-20; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; IgG, immunoglobulin G; HRP-conjugated IgG, HRP-labeled goat anti-human IgG gamma chain; Ch+, Chagas-positive; Ch–, Chagas-negative; LD, limit of detection; TPH, total parasite homogenate; IP, isoelectric point; Q1, chimeric protein gathering RP1 and RP2, homologous to FRA and SAPA, respectively; pL, $(Lys)_3$; pL-Q1, chimeric protein bearing $(Lys)_3$-RP1-RP2; TRX, thioredoxin residue; TRX-Q1, chimeric protein with TRX next to the RP1-RP2 peptide; TRX-pL-Q1, chimeric protein bearing TRX-$(Lys)_3$-RP1-RP2; K, lysine; LB, Luria–Bertani; PCR, polymerase chain reaction; NTA, nitrilotriacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CD, circular dichroism; UV, ultraviolet; FcMe, ferrocenemethanol; SD, standard deviation; ANOVA, analysis of variance; %*D*, dissociation percentage; and *R*, correlation coefficient.

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NH_2 residues were added to the constructions by inserting a 3-lysine residue, pL-, and the fragment thioredoxin, TRX-, respectively.

Widely used diagnostic biosensors often take advantage of the specificity of immunological reactions using the specific Ab or antigen (Ang) to capture the analyte. The Ang–Ab complex formation is then developed by different approaches. As examples, we can mention biosensors to quantify thrombin [10], *Salmonella enteritidis* infection [11], and Ebola virus infection [12].

Using Ang–Ab reactions, our group and others have recently reported on the use of biosensor technology to diagnose Chagas' disease (also known as American trypanosomiasis) [13–15]. This infection is caused by the parasite *Trypanosoma cruzi*. However, to the best of our knowledge, none of the mentioned attempts have resulted in a ready-to-use device. Two main reasons have contributed to this problem: the lack of reproducibility and unspecific reactions. The latter are caused by cross-reactivity with nonspecific Abs when testing sera of patients suffering from related parasitosis such as leishmaniasis [13,14].

Chagas' disease currently affects approximately 16 million individuals in Latin America alone, with an extra 100 million people considered to be at risk for getting infected [16–18]. The high incidence of the infection and the enormous amount of people at risk [19] encouraged us to continue searching for better analytical tools to provide a reliable chagasic infection diagnosis. The new methodology should be useful to monitor inhabitants of endemic regions as well as to help prevent the nonvectorial manner of disease transmission (mother to child, transfusional, and organ transplantation). Nonvectorial is the most important manner of transmission in nonendemic areas because many infected people have migrated from the countryside to distant cities [20–22].

T. cruzi infection is currently diagnosed by using three main serological methods: enzyme-linked immunosorbent assay (ELISA), indirect hemagglutination (IHA), and indirect immunofluorescence (IIF) [17,23]. Even when these serological immunoassays have demonstrated to be valuable diagnostic tools, their key drawback is that they often give false-positive or undetermined outcomes. This is a major problem for blood banks because it leads to unnecessary disposal of whole blood reservoirs [24]. It has been demonstrated that the reason why the conventional serological reactions may fail is related to the Angs used in the immunoassays, which often display cross-reactions with Abs produced during the course of other illnesses [24]. It has also been suggested that the nature of the Angs, usually a complex protein mixture obtained by disruption of parasites, may lead to difficulties in standardizing kit manufacture [24]. To solve these problems, many articles have reported on the use of recombinant Angs that allow specificity and reproducibility to be enhanced [25–27]. The fusion of DNA sequences encoding antigenic proteins is a promising tool to design new epitopes to be used as sensitizing elements in serological tests. In addition, the use of several antigenic determinants grouped in the same molecule to assist the equilibrated adsorption of the Ang to ELISA plaques has been described, thereby facilitating standardization during reagent production [28,29]. Moreover, it was recently demonstrated that sensitizing immunological devices with fused recombinant peptides yield higher signals than those obtained when using assortments of those peptides [30]. Having taken into account our previous results showing that conflictive sera from patients suffering from leishmaniasis presented cross-reactions when using biosensors exposing raw parasite proteins [14], we designed new chimeric proteins to be used as sensitizing elements. These proteins gather two recombinant peptides, RP1 and RP2, in the same molecule. RP1 and RP2 are homologous to the *T. cruzi* flagellar repetitive antigen, FRA, and the shed acute-phase antigen, SAPA, respectively. These two peptides have already demonstrated their individual specificity when used separately for Chagas' disease diagnosis by ELISA [27,31]. In addition, FRA and SAPA have been described as highly antigenic, a convenient attribute because a relatively large amount of their specific Abs is expected to be found in sera from infected patients, thereby yielding better signals than other less antigenic molecules [26,27,32].

One more drawback of the serological methods currently used to diagnose chagasic infection is that they are not suitable to be used in automatic equipment, a desirable feature to count with in blood banks, where a large number of samples must be screened for this disease. To reuse any immunoassay device, it is necessary to dissociate the usually quite stable Ang–Ab complex. With this in mind, we favored the covalent link of the analyte-capturing molecule so that it could be treated under relatively extreme conditions to regenerate the bioreactive layer.

In the current study, we built and assayed biosensors useful to detect *T. cruzi* infection using different biorecognition interfaces that use new tailor-made chimeric proteins designed to greatly increase specificity, to be directionally linked to electrodes and to withstand regeneration procedures. Results were compared with those obtained with a commercially available, currently used Chagas diagnostic ELISA kit. A schematic illustration of the outline of our proposal is presented in Fig. 1.

Materials and methods

Chemicals and reagents

All chemicals were of analytical grade. 3-Mercapto-1-propanesulfonic acid (thiol), 1,3-diisopropylcarbodiimide (carbodiimide), and Tween-20 (T) were supplied by Aldrich. Hydrogen peroxide was obtained from Merck, and all other chemicals were purchased from Sigma. Phosphate-buffered saline (PBS) solution containing 0.16 M NaCl, 0.0027 M KCl, 0.010 M Na_2HPO_4, and 0.0018 M KH_2PO_4 was prepared. The washing solution, PBS-T, was prepared by the addition of the necessary Tween-20 amount to PBS solution to reach a final concentration of 0.5 g L^{-1}. Horseradish peroxidase (HRP)-labeled goat anti-human immunoglobulin G (IgG) gamma chain (HRP-conjugated IgG) was obtained from Zymed. Molecular biology reagents were purchased from Promega unless otherwise stated.

Serum panel

To establish the serological status of the serum samples, we used the criterion recommended by the World Health Organization (WHO) [17]. Accordingly, samples testing positive by two different assays were considered to be Chagas-positive (Ch+), and samples presenting negative results by both tests were considered to be Chagas-negative (Ch–). The commercial kits Chagatest IHA, based on epimastigote total homogenate Angs, and Chagatest ELISA recombinant version 3.0 (both from Wiener Lab, Argentina), were used to confirm the serum sample type. Ch+ samples were obtained from 15 confirmed chronic *T. cruzi*-infected patients, and Ch– control samples were obtained from 20 healthy individuals showing no other proved reactivity.

Specificity of the assay was studied using 68 samples from patients suffering from diseases that have been reported as causative of false-positive results for *T. cruzi* infection [33]. This serum panel was composed of (i) 19 and 8 samples from patients infected with *Toxoplasma gondii* and *Treponema pallidum*, respectively; (ii) 19 and 7 samples from individuals suffering from systemic lupus erythematosus and rheumatoid arthritis, respectively; and (iii) 15 sera from individuals with clinical manifestations of cutaneous leishmaniasis, kindly provided by M.E. Brito from the Centro de Pesquisas Ageu Magalhães, Fundação Oswaldo Cruz (Brazil).

A Ch+ serum pool of 31.0 μg ml^{-1} specific anti-*T. cruzi* IgG, whose concentration was determined as described elsewhere

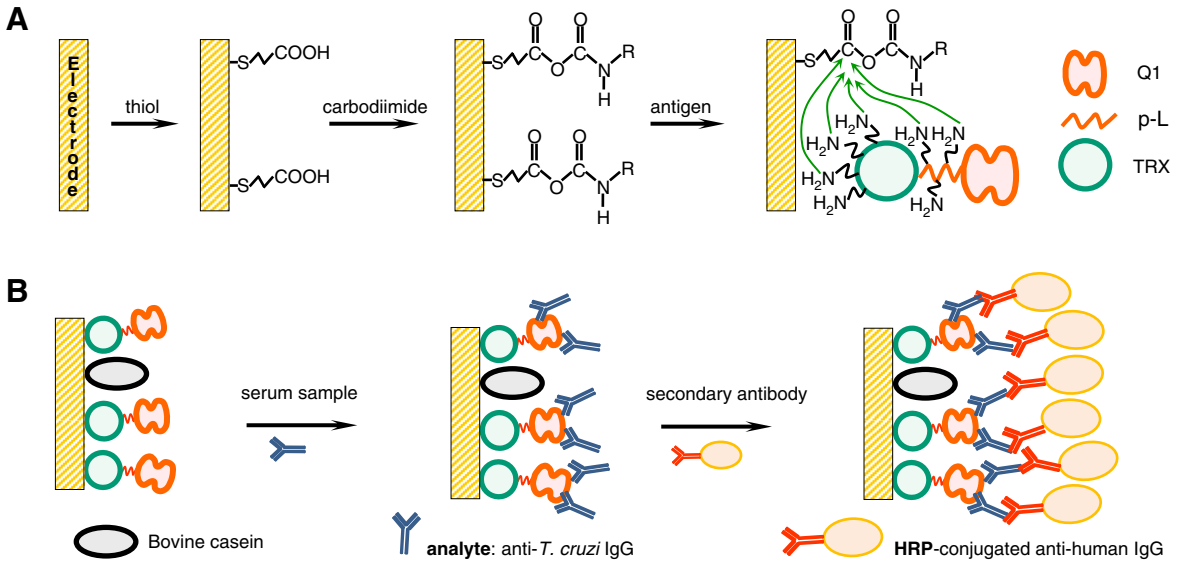

Fig. 1. (A) Schematic representation of the strategy used to orient the recombinant protein: first modification of the electrode with the thiol, then activation of the thiolated surface with carbodiimide, to finally allow the subsequent reaction with the amine moieties of the protein Lys residues. (B) Conceptual scheme of the immunoassay layout.

[14], was used to obtain the limit of detection (LD) of the method. Dilutions of this sample were used to build the calibration plot.

Total parasite homogenate preparation

Epimastigotes of *T. cruzi* (Tulahuen strain) were grown in liver infusion tryptose medium (Cultilab, Brazil), supplemented with 10% fetal calf serum [34]. The epimastigote total parasite homogenate (TPH) was obtained by resuspension of the washed cells in 5 volumes of 1 mM *N*-*p*-tosyl-L-lysine chloromethyl ketone and 1 mM phenyl-methylsulfonyl fluoride in distilled water, freeze and thaw (four cycles), and posterior sonication (20 kHz, 30 W, 2 min). The isoelectric point (IP) of TPH was measured as described in a previous work [14]. The TPH IP ranged between 5.9 and 6.3. Therefore, when trying to favor electrostatic attachment of TPH to the thiol-modified electrodes, the pH of the working solution was regulated to 5.0 to ensure that proteins were positively charged, whereas thiolated surface was positively charged at the same pH [35].

Recombinant protein preparation and characterization

Two different oligonucleotide sequences encoding distinct recombinant peptides, RP1 and RP2, homologous to the previously described *T. cruzi* Angs FRA and SAPA, respectively, were used to build four different chimeric proteins: (i) Q1, which has RP1 and RP2 gathered in the same molecule; (ii) pL-Q1, bearing $(Lys)_3$-Q1; (iii) TRX-Q1, with the fusion vector fragment thioredoxin (TRX) next to the RP1-RP2 peptide; and (iv) TRX-pL-Q1, bearing TRX-$(Lys)_3$-RP1-RP2. The RP1, RP2, and pL-Q1 constructions were deposited in the GenBank under the Accession Numbers FJ440558, FJ440559, and FJ440556 (named as CP1), respectively, and are freely available at http://www.ncbi.nlm.nih.gov/Genbank.

The amino acid sequences of the different parts of TRX-pL-Q1, where lysine (K) residues are highlighted in bold, were as follows:

TRX (with 13 K): MSD**K**IIHLTDDSFDTDVL**K**ADGAILVDFWAEW CGPC**K**MIAPILDEIADEYQG**K**LTVA**K**LNIDQNPGTAP**K**YGIRGIPTLLLF**K** NGEVAAT**K**VGALS**K**GQL**K**EFLDANLAGSGSGHMHHHHHHSSGLVPRG SGM**K**ETAAA**K**FERQHMDSPDLGTDDDD**K**AMADIGSEF. pL (with 3 K): **KKK**.

Q1 (with 2 K): LADRAFLEQ**K**PEGVPLRELPLDDDSDFVAMEQERRQ LLE**K**DPRRNAREIAALEESMNARAQELARELLIGTEAHMDSSSDSSAHST PSTPADSSALSTPSTPADSSAHSTPSTPADSSAHSTPSTPAGHGATGMV-LIL PD.

Escherichia coli cells bearing the plasmid of interest were harvested overnight in Luria–Bertani (LB) medium with 0.1 mg ml^{-1} ampicillin at 37 °C. Plasmidic DNA minipreparations were performed following the procedure described by Sambrook and coworkers [36].

The DNA fragments encoding the desired proteins were amplified by polymerase chain reaction (PCR). The primers used to obtain the different proteins were RP1f (5′-GAATTCAAGAAGCTTGCCGACCGC-3′), RP1r (5′-GAGCTCGCGTGCCAGCTCCTGTGC-3′), RP2f (5′-GAGCT CCTGATTGGCACGGAAGC-3′), RP2r (5′-GTCGACATCGGGCAAAATCA AAACC-3′). To obtain constructions with the $(Lys)_3$ residue, instead of RP1f, the primer RP1f-pL (5′-GAATTCAAGAAGAAGCTTGCCGAC-3′) was used for their amplification. The primers were synthesized by Invitrogen (Argentina).

The expression vector pET24a (Novagene) was used to build Q1 and pL-Q1, whereas the pET-32a plasmid (Novagene) was used to obtain TRX-Q1 and TRX-pL-Q1; this expression vector keeps the TRX fragment [37], which has 13 additional Lys residues. Encoding amplicons were subcloned in the *Eco*RI–*Sal*I site of the vectors pET24a and pET32a to obtain the chimeric constructions RP1 and RP2. Competent bacteria were transformed by one-pulse electroporation (2.5 kV, 25 μF) using a Bio-Rad Gene Pulser (Bio-Rad Laboratories) under the conditions specified by the manufacturer.

E. coli BL21(DE3) cells bearing the different plasmidic constructions were grown overnight in LB medium supplemented with 0.1 mg ml^{-1} ampicillin (pET32a) or kanamicin (pET24a) at 37 °C with agitation. The respective Q1, pL-Q1, TRX-Q1, and TRX-pL-Q1 proteins were induced for 3 h with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG). The soluble extracts were purified with Ni-NTA (nitrilotriacetic acid) columns (GE Healthcare) as described elsewhere [25,30]. Protein quantitation was performed by using Bradford's assay [38]. Purity of the recombinant proteins was analyzed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the electrophoretic bands were stained with Coomassie Brilliant Blue by the Laemmli method [39]. Fig. 2 shows the SDS–PAGE results of the TRX-pL-Q1 purification process using an Ni-NTA column. Circular dichroism (CD) spectroscopy was

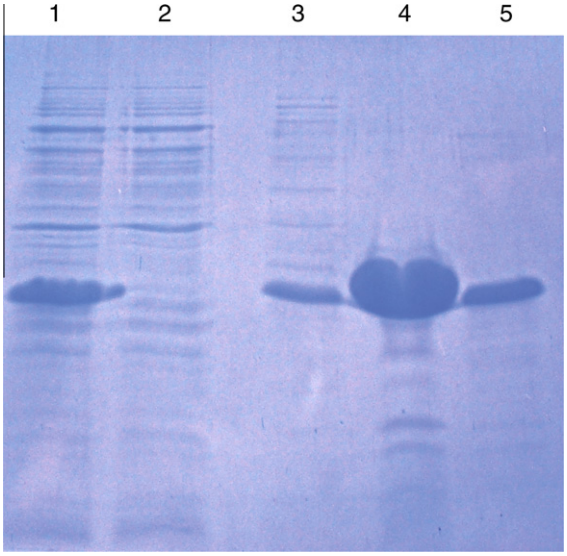

Fig. 2. SDS–PAGE results of TRX-pL-Q1 purification process using a Ni-NTA column. Lane 1, raw extract; lane 2, flow-through eluate; lane 3, 50 mM imidazole eluate; lane 4, 100 mM imidazole eluate; lane 5, 250 mM imidazole eluate.

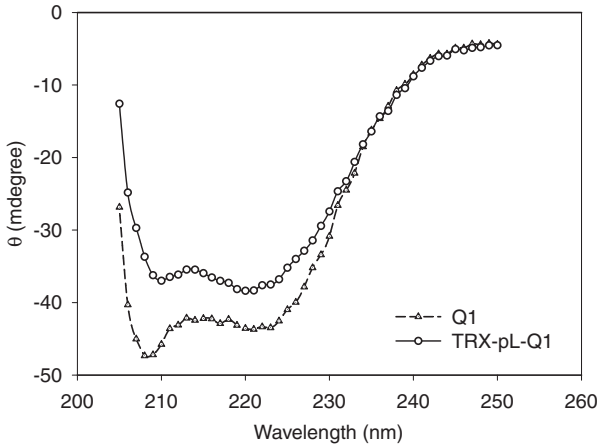

Fig. 3. Far-UV CD spectra of recombinant proteins Q1 (solid line) and TRX-pL-Q1 (dashed line).

used to estimate the secondary structure of Q1 and TRX-pL-Q1. The far-UV (ultraviolet) CD spectra of approximately 70 μg ml^{-1} protein in buffer (50 mM sodium phosphate [pH 7.4] and 100 mM NaCl) were recorded with a Jasco J-8150 spectropolarimeter, operating at room temperature, over the spectral range of 205–250 nm, 1 nm bandwidth, and 20 nm min^{-1} scanning rate. Data were averaged over five accumulations and were corrected by subtracting the baseline recorded using the same quartz cuvette (1 cm path length), buffer, and parameters. Fig. 3 depicts CD spectra results that show regular peaks at 208 and 222 nm, indicating that the conformation of both chimeras is α-helix folded [40].

The IP of all the chimeric constructions was above 5.0, as estimated by the Expert Protein Analysis System (ExPASy) on the ExPASy server [41]. Therefore, electrostatic attachment of the recombinant proteins to the electrode surface was favored by immersing the thiol-modified electrodes into each of the protein solutions at pH 4.7 so that the proteins were positively charged at the working pH.

Biosensor assembly

Gold electrodes were cleaned with piranha solution (18 M H_2SO_4, 0.30 g ml^{-1} H_2O_2, 3:1).[2] Then cyclic voltammetry was performed in 2 M H_2SO_4 (0.050 V s^{-1}, from 0 to +1.700 V vs Ag/AgCl reference electrode).

The clean electrodes were immediately immersed in 10 mM thiol solution in N_2-degassed water and left overnight at 4 °C. After rinsing with distilled water, the thiol-modified electrodes were treated in two different manners: (i) to favor the electrostatic attachment of the Ang, the thiol-modified electrodes were soaked in PBS solution (pH 5.0) [35] containing 0.18 mg ml^{-1} TPH proteins or PBS solution (pH 4.7) containing 0.015 mg ml^{-1} Q1 for 2 h at 37 °C; (ii) to favor the covalent link of the Ang, the thiol-modified electrodes were immersed in 1% (w/v) carbodiimide aqueous solution for 2 h at room temperature. Once rinsed, the electrodes were submerged in the protein solution prepared in 25 mM Hepes buffer at 0.3 pH units above the IP of each sensitizing protein to prevent Ang electrostatic attachment. The protein concentrations were those previously determined as optimal (i.e., 0.18 mg ml^{-1} for TPH), whereas recombinant chimeras ranged between 0.015 and 0.060 mg ml^{-1}. Fig. 1A depicts the proposal to favor the Ang covalent attachment used to build the chimeric sensitized bioelectrodes.

To block unmodified sites, the Ang-sensitized biosensors were exposed to 10 mg ml^{-1} bovine casein sodium salt in PBS for 10 min at 37 °C. After rinsing thoroughly with PBS-T, the devices were stored at 4 °C in PBS (pH 7.2) until use in immunoassays.

Immunoassay

The bioelectrodes were incubated for 30 min at 37 °C in the convenient sample dilution, usually 1:200 in PBS (pH 7.2), except when the calibration plot was constructed and the LD was determined, where dilutions varied between 1:10 to 1:5000. After being rinsed three times with PBS-T, biosensors were immersed for 30 min in HRP-conjugated IgG diluted 1:1500 in PBS solution (pH 7.2) at 37 °C. Fig. 1B shows a scheme of the immunoassay layout. After rinsing, the devices were kept at 4 °C in PBS (pH 7.2) until performing the electrochemical experiments.

Electrochemical measurements

All electrochemical measurements were performed in a three-electrode cell with the self-assembled biosensor as the working electrode, an Ag/AgCl as the reference electrode, and a gold flag counter electrode. Amperometric measurements and cyclic voltammetries were recorded with an Autolab electrochemical analyzer equipped with PGSTAT 30 software.

Amperometric experiments were performed at 25 °C and 0.080 V (vs Ag/AgCl). Once the cell containing the soluble mediator, 3.0 mM ferrocenemethanol (FcMe) solution in PBS, reached the equilibrium, the current value was recorded as i_b. Then H_2O_2 solution was added to reach a final concentration of 1.8 mM. The current, i_s, was recorded until stabilization (generally for 4 min). The signal, catalytic current, i_c, was calculated as $i_c = i_s - i_b$. The catalytic cycle that originates the signal is described by

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

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

Bioreactive layer regeneration

Experiments to evaluate different reagents and strategies to detach the analyte from the bioreactive layer were performed because

[2] Safety measures should be used with this highly corrosive reagent.

the best detaching agent should be determined for each particular Ang–Ab system [42]. Biosensors assembled with the covalently attached TRX-pL-Q1, the chimeric construction that rendered the best signal-to-noise ratio, were used to perform immunoassays using a Ch+ serum sample. The already used biosensors were subjected to chemical treatment with different chaotropic agents, namely H_2O, 0.1 M glycine–HCl (pHs 1.7, 2.0, and 2.8), 0.1 M NaCl in HCl (pH 2.0), and 3.5 M KSCN in PBS (pH 6.6). Two different protocols were carried out, depending on whether batch or continuous flow treatment was assayed. In the case of stationary treatment, the bioelectrodes were immersed in the detaching solution for different time periods ranging from 10 to 40 min. When the continuous flow strategy was used, experiments were performed using a 602 S Watson–Marlow pump set at a pumping rate of 20 ml min^{-1}.

Biosensor reusability experiments

The regenerated TRX-pL-Q1 biosensors were used to perform 14 consecutive immunoassays using, alternatively, one Ch+ and one Ch– sample after each regenerating step to verify the complete detachment of the analyte. The reusability working protocol was applied cyclically as follows. A set of 20 biosensors was assembled, covalently linking TRX-pL-Q1 according to the "Biosensor assembly" section above. Of these bioelectrodes, 10 were used to test a 1:200 dilution of a Ch+ serum sample, whereas the 10 remaining biosensors were used to test a 1:200 diluted Ch– sample. After incubating the 20 bioelectrodes with HRP-conjugated IgG, the electrochemical experiments were performed as described in the "Electrochemical measurements" section above. The 20 already used bioelectrodes were treated with glycine–HCl (pH 2.8), the most effective detaching agent, either at constant flow and room temperature or in batch at 37 °C, for 30 min. In the following cycle, those 10 biosensors that had been used with the Ch+ sample were reused to assay the Ch– sample and vice versa.

ELISA

For method comparison, the commercial Chagatest ELISA recombinant version 3.0 (Wiener Lab) was used. In these assays, 1:20 dilutions of the samples were used according to the manufacturer's instructions. The results, reported as optical densities (OD_{450}), were obtained with an LD400 luminescence detector (Beckman Coulter) operating with a 450-nm filter.

Results are expressed as means ± standard deviations (SDs). A Student's *t* test was used for statistical comparisons prior verification of the normal variance distribution. One-way analysis of variance (ANOVA) followed by the Fisher's test was used for multiple comparisons. The level of significance was set at $P < 0.05$.

Parameters used to describe biosensor performance

The cutoff value was estimated as the mean i_c value of 20 different Ch– serum samples assayed in duplicate (2 bioelectrodes for each sample) plus three times the SD. Samples rendering signals above or below the cutoff were considered to be Ch+ or Ch–, respectively. Samples producing signals ranging within the cutoff value ±10% were considered to belong to the undetermined zone.

LD was evaluated as the minimum concentration producing the smallest signal distinguishable from the background with a reasonable certainty (95% in our case) [43]. Therefore, LD was assessed as the anti-*T. cruzi* IgG concentration rendering the minimum i_c value above the cutoff value.

Specificity was expressed as 100 times the detectability index of negative samples, with this latter parameter being defined as the number of Ch– samples rendering i_c below the cutoff value over the total number of Ch– samples evaluated.

The signal-to-noise ratio of biosensors assembled with the different Angs was calculated as the respective mean signal, $\bar{i}_c$, over their cutoff values. $\bar{i}_c$ was calculated from measurements of 20 replicate biosensors assembled with each Ang when assaying the same Ch+ sample.

The detachment efficiency of the different reagents and regeneration procedures was evaluated using the dissociation percentage (%*D*), calculated as

$$\%D = \frac{i_c - i_r}{i_c} 100,$$

where i_c and i_r are the signals measured before and after the regeneration step, respectively.

Results and discussion

Analyte-capturing Ang: chimera construction and primary evaluation

In a previous work, we demonstrated the feasibility of using an amperometric biosensor assembled with electrostatically attached *T. cruzi* TPH proteins to assess the presence of specific IgG anti-*T. cruzi* in serum samples from *T. cruzi*-infected patients [14]. However, those biosensors showed false-positive results due to cross-reactions when testing sera from patients suffering from a Chagas' disease-related parasitosis, namely leishmaniasis. To increase specificity, biosensors were assembled using new synthetic receptor layers containing the recombinant protein Q1 that gathers RP1 and RP2 homologous to FRA and SAPA, respectively. Whereas specific IgG anti-RP1 homologous has been found in 99.6% of serum from chronic patients [32], specific IgG anti-RP2 homologous has been reported to be present in serum from acute-infected individuals [44,45]. Therefore, the new RP1-RP2-fused peptide chimera was conceived to be, in principle, useful to react with anti-*T. cruzi* IgG Abs present in serum of patients at the acute or chronic stage of the infection.

Q1 optimal concentration to sensitize electrodes was assessed (5 replicates for each concentration) and turned out to be 0.015 g ml^{-1} ($P < 0.01$) (results not shown).

To initially evaluate Q1 specificity performance, we built biosensors using Q1 and TPH. The sensitizing proteins were attached by favoring electrostatic forces because this procedure had rendered the highest signals in our previous work [14]. Preliminary immunoassays using amperometric detection were performed for three potentially conflictive leishmaniasic sera that had shown cross-reactions when performing conventional ELISA with microplates sensitized with TPH. Results showed 100% and 33% specificity, evaluated as detailed in "Parameters used to describe biosensor performance" section above, for biosensors assembled with Q1 and

Table 1
Catalytic current, i_c, recorded when testing 1:200 dilutions of the same Ch+ serum sample with biosensors built by covalently linking the different antigens.

Antigen	i_c (μA cm^{-2})	Cutoff[a]	Signal-to-noise[b]
Q1	2.4 ± 0.2*	0.43	5.6
pL-Q1	2.6 ± 0.2*	0.44	5.9
TRX-Q1	3.0 ± 0.3*,#	0.49	6.1
TRX-pL-Q1	3.3 ± 0.3*,#	0.51	6.5
TPH	4.0 ± 0.4	0.64	6.3

Note: Results are expressed as mean signals ± SDs of 20 replicates.
[a] The cutoff values were calculated as means plus three times the SDs obtained when performing immunoassays with 1:200 PBS dilutions of 20 different Ch– samples.
[b] Mean signal divided by the cutoff.
* $P < 0.05$ vs TPH.
# $P < 0.05$ vs Q1 and pL-Q1.

Table 2
Signals obtained with TRX-pL-Q1 biosensor and ELISA Chagatest recombinant version 3.0 when testing several dilutions of the same Ch+ serum sample containing 31 μg ml^{-1} specific IgG.

Specific IgG concentration (μg ml^{-1})	Biosensor signal (μA cm^{-2})	ELISA signal (OD)
3.100	1.7 ± 0.2	2.5 ± 0.2
0.620	1.7 ± 0.1	1.2 ± 0.2
0.484	1.6 ± 0.1	0.5 ± 0.1
0.242	1.35 ± 0.09	0.042 ± 0.002[a]
0.121	0.79 ± 0.09	0.044 ± 0.002[a]
6.20 × 10^{-2}	0.53 ± 0.06	0.043 ± 0.002[a]
3.10 × 10^{-2}	0.36 ± 0.05[a]	0.044 ± 0.002[a]

Note. Results are expressed as mean signals ± SDs of 3 replicates.
[a] Signal below the cutoff value. The cutoff value was calculated as the mean signal plus three times the SD obtained when performing tests with 20 different Ch– samples.

TPH, respectively (4 replicates for each Ang studied). This clearly indicates enhanced specificity when using Q1.

To compare sensitivities, we assembled biosensors with Q1 and TPH by favoring electrostatic interactions. Immunoassays were carried out using the same 1:200 dilution of a Ch+ serum sample (4 biosensor replicates for each Ang). The i_c values obtained were 4.2 ± 0.3 and 2.5 ± 0.2 μA cm^{-2} for TPH- and Q1-assembled biosensors, respectively. This indicates that a signal loss of approximately 40% occurred when using Q1. This is the expected phenomenon when changing TPH proteins by Q1 in the bioreactive layer because, in the first case, the different anti-*T. cruzi* IgGs present in the sample may be captured at the biosensor surface, whereas when using Q1 the only specific IgGs bound are those that react with RP1 or RP2 epitopes. Considering the signal decrease when using Q1, it became imperative to improve the Ang–Ab binding efficiency to capture most of the target analyte so as to enhance sensitivity.

Sensitivity improvement by directional link of the Ang

When assembling the biosensor giving preference to electrostatic interactions, the Ang molecule does not necessarily attach to the electrode surface at an optimal orientation, so that the blockage of several specific binding sites may occur. Therefore, we attempted to direct the linkage through facilitating covalent unions between the electrode surface and the Ang sites not involved in the recognition reaction. With this purpose, the thiol-modified surface of the electrode was activated with carbodiimide to facilitate the nucleophilic substitution via the Ang NH_2 residues. We designed pL-Q1, which gathered the same epitopes as Q1 but added a 3-lysine residue located adjacent to the highly antigenic epitopes. In this way, the new Ang had an increased number of free NH_2 residues to react with the activated surface. It should be noted that Q1 has only 2 lysine moieties that could compete with those in the pL fragment for substitution reaction with carbodiimide-activated surface.

The signals obtained when assaying 1:200 dilutions of the same Ch+ serum sample with biosensors assembled with Q1 and pL-Q1 (6 replicates each) were 2.4 ± 0.2 and 2.7 ± 0.2 μA cm^{-2}, respectively ($P < 0.05$). Even though i_c rose 13% for devices using pL-Q1, the signal loss was still significant, as compared with that obtained when sensitizing the electrodes with TPH. One possible reason for this is that the reaction efficiency between the Lys-NH_2 residues and the carbodiimide-activated electrode surface might not be optimal because of some steric hindrance due to Q1 size, which is much bigger (140 amino acids) than the 3-Lys residue that had been inserted. To evaluate this hypothesis, and in an attempt to enhance the oriented Ang-linking strategy, we included additional NH_2 residues incorporating a protein fragment whose size was on the same order as that of Q1. The proposed alternative was to change the protein expression system pET-24a by pET-32a because, in this latter case, the 167-amino-acid residue TRX, bearing 13 extra Lys residues, remains as part of the expressed protein. Fig. 1A depicts the strategy used to direct the covalent Ang attachment. In addition, it has already been demonstrated that proteins expressed using pET-32a show no unspecific reaction for the TRX moiety [37]. Therefore, we built two new chimeric constructions, TRX-Q1 and TRX-pL-Q1, to assemble new bioelectrodes.

Table 1 shows the signals obtained when performing immunoassays with bioelectrodes built with the different Angs assayed, testing 1:200 dilutions of the same Ch+ serum sample ($n = 20$ for each Ang). The cutoff values obtained for 20 different Ch– serum samples, and their respective signal-to-noise ratios calculated as described in the "Parameters used to describe biosensor performance" section above, are also shown in Table 1. The ANOVA test indicated that at least two of the mean signals recorded with biosensors built with the Angs studied were significantly different ($P < 0.05$). The Fisher's test for multiple comparisons showed that the mean value of those groups lacking TRX was significantly lower than that of groups having TRX at the 95% level of significance, thereby indicating that the presence of the TRX residue in the chimeric construction actually favored a proper epitope exposure to react with the analyte. This suggests that the proposed strategy of oriented bioreceptor attachment to the electrode was successful and that the 167-amino-acid peptide chain TRX did not hinder the analyte binding to the sensitized surface.

The results depicted in Table 1 also indicate that, even though the mean signal of the biosensors assembled with TRX-Q1 and TRX-pL-Q1 (3.0 ± 0.3 and 3.3 ± 0.3 μA cm^{-1}, respectively) could not be statistically distinguished, there is a tendency suggesting that proteins having a pL moiety exhibit higher signals than those of their analogues lacking pL. Taken together, these results indicate that the higher the number of extra NH_2 residues, the higher the signal, in agreement with the oriented attachment assumption. To confirm this, we compared the performances of biosensors built by facilitating electrostatic interactions with those of biosensors built by covalently attaching TRX-pL-Q1. The signals were 2.7 ± 0.3 and 3.3 ± 0.3 μA cm^{-1}, respectively ($P < 0.01$, $n = 10$). These results reinforce our hypothesis that the directed covalent attachment of the bioreactive molecule via sites not involved in the Ang–Ab reaction enhances the biosensor sensitivity.

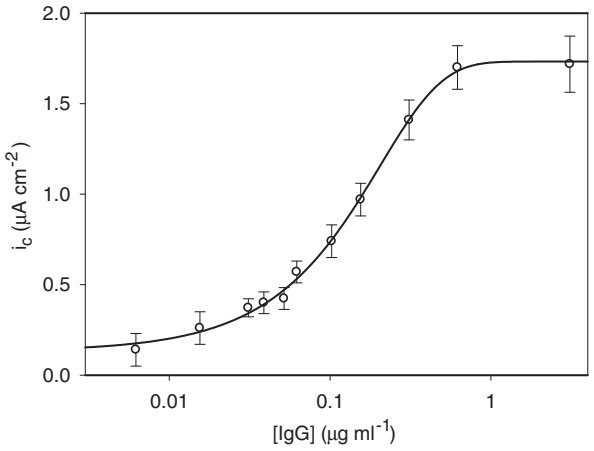

Fig. 4. Calibration plot depicting the signals recorded with biosensors built with TRX-pL-Q1 as a function of specific IgG concentration: catalytic current at 0.080 V constant voltage vs Ag/AgCl, in 3.0 mM FcMe, with 1.8 mM H_2O_2 in PBS, for series of 5 electrodes at each concentration (medium ± SD).

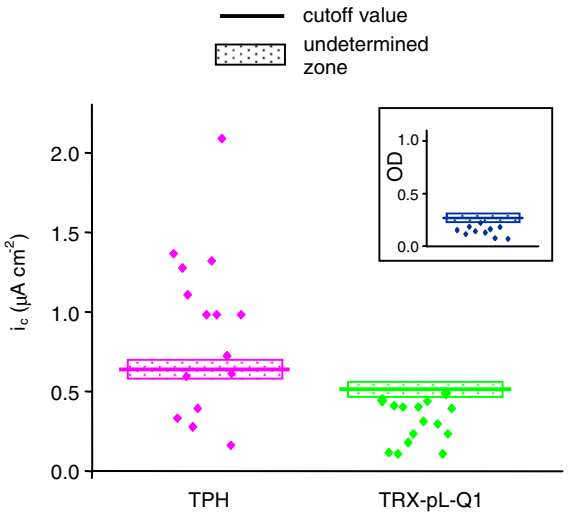

Fig. 5. Comparison of the catalytic current obtained when testing 15 leishmaniasic samples with biosensors built with TPH and the recombinant protein TRX-pL-Q1. Inset: optical density obtained with a commercial recombinant ELISA kit when analyzing 10 leishmaniasic samples.

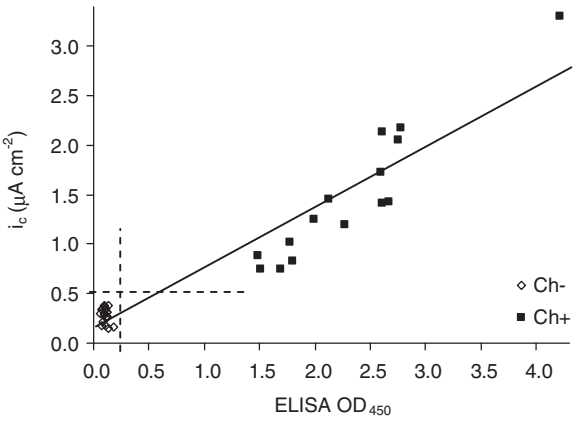

Fig. 6. Correlation curve between the signals recorded using biosensors built with the recombinant protein TRX-pL-Q1 and those obtained with a currently used commercial ELISA kit. The horizontal and vertical dotted lines indicate the respective cutoff values.

Although biosensors built with TPH rendered the greatest signal (4.0 ± 0.4 vs 3.3 ± 0.3 μA cm^{-1} for TRX-pL-Q1-built electrodes), the highest signal-to-noise ratio (6.5) was reached when using the latter one, a value even better than that obtained with TPH (6.3) (see Table 1). This is an important feature displayed by TRX-pL-Q1 because its aptitude to discriminate Ch+ from Ch– samples turned out to be more efficient than that exhibited by TPH.

The calibration plot obtained when performing immunoassays with biosensors built with TRX-pL-Q1 is shown in Fig. 4. Each point represents the medium of the signals recorded with 5 biosensor replicates.

Specificity of the assay

To assess the performance of the biosensors built with the proposed chimeric construction, we performed immunoassays of 68 samples (see details in "Serum panel" section above) from individuals with diseases reported as potentially interfering when diagnosing chagasic infection [33]. Chimera- and TPH-sensitized biosensors rendered no false-positive results when testing samples from patients with diseases other than leishmaniasis. Fig. 5 displays the signals obtained with biosensors built with TPH and TRX-pL-Q1 when assaying the 15 conflictive leishmaniasic sera. TPH biosensors displayed cross-reactions leading to 40% specificity, evaluated as described in the "Parameters used to describe biosensor performance" section above. On the other hand, the specificity obtained with biosensors assembled with the new chimeric construction rose to 100% for the same serum samples. Moreover, in Fig. 5, it can also be seen that TPH-sensitized devices produced 33% of the results below the cutoff value belonging to the undetermined zone, whereas TRX-pL-Q1 biosensors produced only 7% of doubtful results, thereby demonstrating an improved performance.

Method comparison

To validate the proposal, we performed immunoassays by two alternative methods: biosensors built with TRX-pL-Q1 and an extensively used commercial ELISA kit sensitized with recombinant proteins (Chagatest version 3.0, Wiener Lab). The serum panel used contained 15 Ch+ and 20 Ch– control samples confirmed to be Chagas-positive and -negative, respectively. Fig. 6 shows the correlation curve obtained when comparing both methods using the serum panel studied. The catalytic current plotted is the mean of 2 replicates measured when testing 1:200 dilutions of 15 Ch+ and 20 Ch– serum samples, as compared with the mean OD_{450} obtained for 2 replicates when testing 1:20 dilutions of the same serum samples with a commercial ELISA kit (dilution as indicated by the supplier in the working protocol).

The calculated correlation coefficient, *R*, was 0.9523, a value that clearly indicates there is good correlation between both methods. It must be pointed out that all of the positive samples rendered currents above the cutoff value (0.51 μA cm^{-2}), whereas all of the negative samples rendered values lower than this, thereby indicating the effectiveness of discriminating the infection status when using the new approach.

Taking into account that, in the case of *T. cruzi* infection, the mere presence of specific Abs indicates exposure to the parasite and consequently that the patient is infected, the detection of the analyte presence is more important than quantifying it. Accordingly, the foremost attribute of a new methodology to diagnose chagasic infection is related to its ability to lower the LD for the specific Abs. The LD of the new approach was compared with that of one commercial recombinant ELISA kit, testing successive dilutions of a 31.0-μg ml^{-1} IgG-specific Ch+ serum pool. The comparative signals obtained by both methods are shown in Table 2. The LDs were 484 and 62.0 ng ml^{-1} when using the commercial ELISA kit and biosensors assembled with TRX-pL-Q1, respectively, thereby indicating that biosensors performed better than the commercial ELISA kit for the serum pool analyzed.

Reusability of the device

We assessed the number of times that TRX-pL-Q1 biosensors can be reused with negligible sensitivity loss as a means of evaluating the eventual cost per determination and the potential of the device to be used in automatic equipment. Table 3 shows the dissociation percentages, *%D*, obtained as detailed in the "Parameters used to describe biosensor performance" section, for the different reagents studied (6 replicates for each one). The best results were obtained when treating biosensors with either 0.1 M glycine–HCl (pH 2.8) or 3.5 M KSCN (pH 6.6). Although both agents seemed to render equivalent *%D* values, it still remained uncertain whether a complete separation of the specific anti-*T. cruzi* IgG from the bioreceptor was achieved. Indeed, the decrease of the signal measured after treating the used biosensors with the agent under study could

Table 3
Dissociation percentages of biosensors assembled with the recombinant protein TRX-pL-Q1 after the treatment with different detaching agents.

Detaching agent	%D[a]
H_2O	34 ± 3
0.1 M glycine–HCl (pH 2.8)	88 ± 3
0.1 M NaCl (pH 2.0)	78 ± 3
3.5 M KSCN (pH 6.6)	87 ± 4

Note: Results are expressed as means ± SDs of 6 replicates.
[a] *%D*, the dissociation percentage, was evaluated as 100 times the difference between the catalytic current measured before and after the treatment divided by the catalytic current recorded before the treatment.

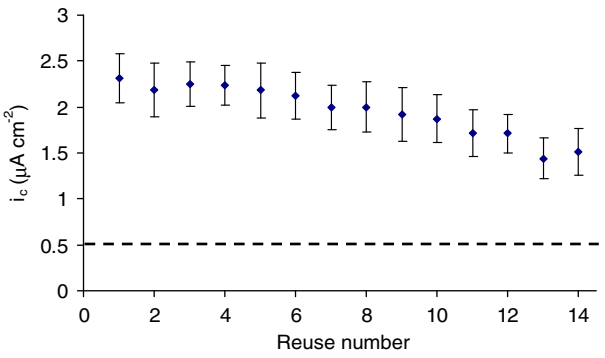

Fig. 7. Signals recorded with the same biosensor for immunoassays performed consecutively once having detached the analyte using the same Ch+ sample. The horizontal dotted line indicates the cutoff value.

have been the result of having removed the secondary Ab, HRP-labeled anti-human IgG, or just having denaturalized the HRP enzyme. Considering this, we performed consecutive immunoassays using the same biosensor to first assay a Ch+ sample and, once regenerated, to assay one Ch– sample. When the recorded signals changed from those correspondent with the Ch+ sample (2.3 ± 0.3 μA cm^{-2}) to values similar to those recorded for the Ch– studied (0.30 ± 0.08 μA cm^{-2}), the analyte detachment was regarded as accomplished. The i_c obtained for the Ch– sample with biosensors regenerated with 0.1 M glycine–HCl (pH 2.8) (6 replicates) was 0.3 ± 0.1 μA cm^{-2}, whereas the same signal recorded when regenerating biosensors with KSCN was 0.8 ± 0.1 μA cm^{-2}. This demonstrates that KSCN was not an efficient detaching agent for our particular Ang–Ab system because the analyte did not completely break free from the bioreactive layer.

The effect of other variables, such as differential shifting of the Ang–Ab equilibrium by flowing the detaching agent, pH, and the incubation temperature, was also studied (results not shown). The whole set of ancillary experiments to determine the optimal assay conditions showed evidence that the analyte detachment was optimal when working with 0.1 M glycine–HCl (pH 2.8) at a washout flow rate of 20 ml min^{-1} at room temperature for 5 min. The results obtained were equivalent when treating the used biosensors with the same reagent in batch at 37 °C for 40 min. Under any of these conditions, the dissociating percentage was 88 ± 3% ($n = 6$).

The number of times that biosensors could be reused after regeneration with the established best protocol was determined. Fig. 7 shows the signals recorded in 14 consecutive tests of the same Ch+ sample using regenerated TRX-pL-Q1 biosensors ($n = 10$). The whole set of values obtained when testing the Ch– sample was always well below the cutoff (data not shown in this figure).

The results demonstrate that biosensors assembled favoring TRX-pL-Q1 covalent attachment to the electrode surface can be reused 10 consecutive times, keeping approximately 80% of the original signal when the same sample is tested. This feature confers the new device the potentiality to be used in automatic equipment. The stable new chimeric protein was efficiently retained on the surface of the electrode and, therefore, withstood the severe treatment to remove the analyte to be further reused, thereby allowing operating cost reduction.

Conclusions

Amperometric biosensors with a new interface that proved to effectively expose the bioreactive molecule have been assembled. The layer contains new chimeric proteins useful for the diagnosis of *T. cruzi* infection. The recombinant molecule that performed better included the TRX residue and a 3-Lys chain, positioned adjacent to the molecule region that specifically interacts with the analyte. The high content of NH_2 groups of TRX and poly-Lys fragments allowed the directed covalent link of the molecule, leading to sensitivity enhancement. The new biosensor could be subjected to severe chemical treatment to detach the analyte. This allowed it to be reused for 10 consecutive immunoassays, thereby demonstrating the potential utility for automated analysis.

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