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Letter

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Trypanosoma cruzi Virulence Factors for the Diagnosis of Chagas' Disease

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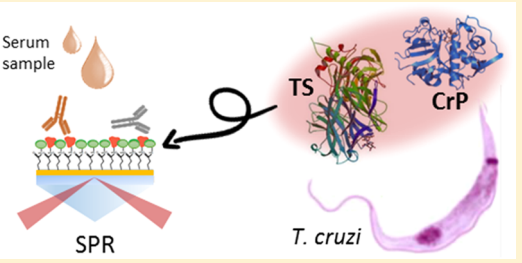
Supporting Information

ABSTRACT: *trans*-Sialidase and cruzipain are important virulence factors from *Trypanosoma cruzi*, the etiological agent of Chagas disease, that have highly antigenic domains in their structure and were reported as potential tools for diagnosis of the illness. The aim of the present study is to assess the possibility of using cruzipain and the catalytic domain of *trans*-sialidase in a Surface Plasmon Resonance-based immunosensor for the diagnosis of chronic Chagas disease. Immunoassays carried out with canine sera verified that cruzipain allows the detection of anti-*Trypanosoma cruzi* antibodies whereas recombinant *trans*-sialidase did not yield specific detections, due to the high dilutions of serum used in the immunoassays that hinder the possibility to sense the specific low titer antibodies. The developed cruzipain-based biosensor, whose price per assay is comparable to a commercial enzyme-linked immunosorbent assay (ELISA), was successfully applied for the rapid quantification of specific antibodies against *Trypanosoma cruzi* in fresh human sera showing an excellent agreement with ELISA.



KEYWORDS: *cruzipain, trans-sialidase, Trypanosoma cruzi, Chagas' disease, diagnostics, Surface Plasmon Resonance*

Chagas' disease (CD), also known as American trypanosomiasis, is a parasitic illness caused by the protozoan *Trypanosoma cruzi* (*T. cruzi*). The transmission of CD is widespread from the limit of Mexico and USA in North America to Chile and Argentina in the south of the continent, representing a serious public health problem. Most human infections in the endemic areas occur through contact with infected bloodsucking insects of the triatomine species; the possibility of transmission through blood transfusion, organ transplant, and congenital infection also exists.[1] Due to population migrations, CD has crossed the borders of America to settle in non-endemic countries,[2] causing globally more than 45,000 deaths per year and approximately 17 million infected people worldwide, according to recent estimations.[3,4]

The diagnosis of CD is based in direct parasitological methods aimed at the confirmation of the presence of the parasite in the patient and in serological tests with the purpose of detecting specific anti-*T. cruzi* antibodies in the serum. At present, the most widely used serological methods are indirect hemagglutination (IHA), indirect immunofluorescence (IIF), and enzyme-linked immunosorbent (ELISA) assays that are based on total homogenates or semipurified antigenic fractions from the parasite, tests which might cross-react with other infections and show contradictory or inconclusive results, mainly related to the characteristics of the antigens.[5,6] Among the available molecular tools,[7] quantitative real-time polymer-

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ase chain reaction (qPCR) has opened new opportunities for detection and quantification of parasitic loads in peripheral blood samples,[8] becoming an accurate surrogate biomarker for diagnosis and treatment monitoring for patients with CD. In the past 20 years, several diagnostic tests using recombinant *T. cruzi* proteins and synthetic peptides[9−13] have proven to provide markedly improved specificity compared to the conventional methods. However, in many cases, these initiatives suffer from limited sensitivity,[14] and better-quality serological assays are still needed.[15]

In order to avoid non-specific reactions and decrease the background in immunological assays, a common practice in diagnostic laboratories is to heat inactivate serum samples.[16] This pretreatment has been found to have a positive[17] or negative[18,19] impact, depending on the serological method. Regarding CD, some *in vitro* diagnostic manufacturers recommend, in package insert instructions, not to use heat-inactivated samples for anti-*T. cruzi* IgG assays as it can lead to misinterpretation of the results. Nevertheless, this practice is usual in some laboratories,[20] and the potential effect of heat inactivation of serum in the diagnosis of CD has not been published.

Although highly utilized in the clinical medicine routine, the conventional assays are time-consuming and require specialized laboratory equipment and trained laboratory personnel. The need to overcome these disadvantages has promoted the development of diagnostic devices in the area of biosensors, intended to combine robustness, simplicity, and portability with adequate specificity and sensitivity. One of the most promising methods is based in the detection of Surface Plasmon Resonance (SPR). In this technique, binding events to a functionalized surface are followed in real time through changes in the refractive index, i.e., without using additional probe molecules.[21] The main advantages of SPR are speed, label-free direct sensing, and the possibility to be applied to remote or resource-limited locations in the point-of-care format.

In recent years, several reports have demonstrated the progress of SPR sensing in medical diagnostics.[22] The feasibility to adopt this technology in clinical chemistry depends on, among others, its cost-effectiveness in relation with existing methods such as ELISA. While the cost per assay of the current SPR detections cannot be lowered to values below commercial ELISA, this technology could gain an impact in cases where the rapid action of clinicians would be required and in situations in which ELISA may be too costly to run.[22]

trans-Sialidase (TS)[23] and cruzipain (CrP)[24] are important virulence factors from *T. cruzi*[25,26] involved in the host−parasite relationship that have highly antigenic domains in their structure.[27] Interestingly, antibodies against TS have been reported as a potential tool for the diagnosis of acute CD,[28] and the catalytic domain of the enzyme can also be used for the diagnosis and monitoring of chronic CD treatment.[29] CrP, the major cysteine proteinase of the parasite, has been proven as a useful tool for the differential diagnosis between chronic CD and other infections.[30]

Among the reported SPR clinical biosensors for medical diagnostics,[22] a total parasite homogenate-based SPR sensor for the detection of anti-*T. cruzi* antibodies in serum samples has been developed from bare gold sensor chips.[31,32] As discussed above, the use of whole or semipurified extracts of *T. cruzi* in the diagnostic tests introduces a source of variability in the assays, yielding controversial results on several occasions.[5,6] Bearing this in mind, CrP and TS as immobilized ligands in SPR-based immunoassays appears as an interesting strategy to investigate the improvement of the sensing of the desired antibodies.

The aim of the present study is to assess the possibility of using CrP and the catalytic domain of TS in a SPR-based immunosensor for the diagnosis of chronic CD. The obtained biosensors are first evaluated, as a proof-of-concept stage, using pools of canine sera infected and non-infected with *T. cruzi*. The CrP-based biosensor, which was proven to have the capability to sense the antibodies of interest, is applied to the analysis of clinical samples, i.e., fresh and heat-inactivated human sera. The performance and cost-effectiveness of the CrP-based SPR immunosensor for the diagnosis of CD is discussed in comparison to ELISA.

To our best knowledge, this is the first SPR-based immunosensor for anti-*T. cruzi* detection using purified proteins as recognition elements.

Results and Discussion. *Construction of the Immunosensor.* The success of an immunosensor relies on the choice of an effective capturing molecule, able to recognize quickly and certainly the analyte of interest. Thereby, two *T. cruzi* proteins, TS or CrP, were covalently immobilized onto the SPR sensor chip covered with *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC)/*N*-hydroxysuccinimide (NHS)-activated single 3-mercaptopropionic acid (MPA) self-assembled monolayer (SAM). The immobilization was carried out inside the SPR device, and it was monitored by the shift of the SPR angle ($\Delta\theta_{SPR}$). As shown in Figure 1, the

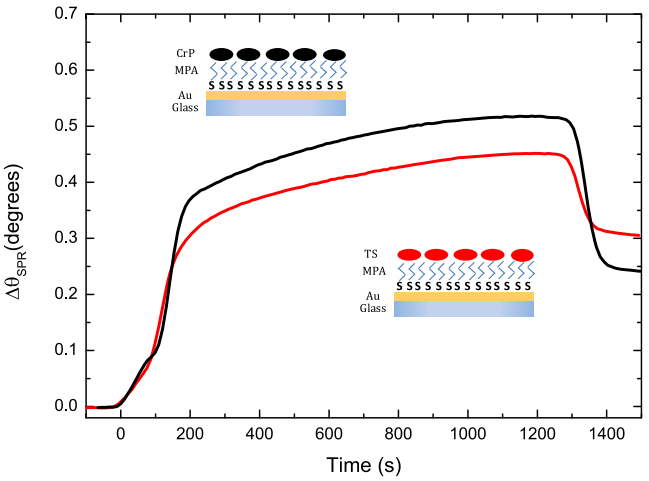

Figure 1. Sensograms resulting from the immobilization of 15 μg/mL TS (red) and 120 μg/mL CrP (black) onto activated 3-MPA SAMs. Inset: schemes of the obtained sensor platforms (not drawn to scale).

injection of *T. cruzi* protein solutions produced a notorious shift in the SPR angle, due to an increase in the refractive index of the medium caused by the anchorage of the proteins to the NHS ester groups on the activated sensor surface.

Net changes in SPR angle can be related to the surface coverage through the *de Feijter* equation (see the Supporting Information). According to this estimation, the coverage of TS and CrP yielded 184 and 148 ng/cm^2, respectively. These values represent 41% (TS) and 35% (CrP) of surface coverage (considering a close packed array as 100% of coverage); a fact that is compatible with an adequate ligand−analyte interaction

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in terms of the availability of capturing sites in the immobilized molecules.

In view of these data, both *T. cruzi* proteins were successfully immobilized onto the activated MPA SAMs.

Proof-of-Concept Stage: SPR Immunoassays for the Detection of Anti-T. cruzi Antibodies in Canine Serum. The performance of the developed sensor platforms in the detection of anti-*T. cruzi* antibodies was preliminarily evaluated by the injection in the SPR device of positive and negative pools of canine sera at 1/250 and 1/400 dilutions in PBS. Figure 2 shows the $\Delta\theta_{SPR}$ that characterizes the interaction between serum samples and Au-MPA-CrP (Figure 2A) and Au-MPA-TS (Figure 2B) sensor platforms.

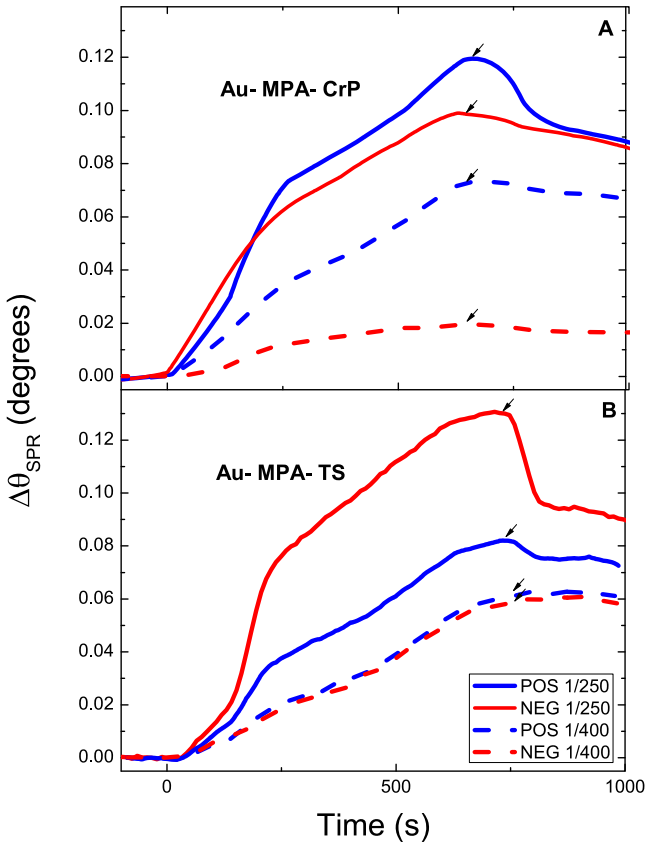

Figure 2. Sensograms corresponding to the 10 min injection of positive (POS) and negative (NEG) pools of canine sera over Au-MPA-CrP (A) and Au-MPA-TS (B) sensor platforms. Arrows point to the beginning of the running buffer (10 mM PBS, pH 7.4) injection that removes the weakly bound species at the immunosensor surface.

As SPR response is directly proportional to the concentration of biomolecules on the surface, the decrease in $\Delta\theta_{SPR}$ as the serum dilution is increased can be ascribed to a lower amount of molecular recognition events. The weakly bound ligands (specific or non-specific) are washed off the surface after each injection of running buffer, while the remaining SPR signal accurately measures strong biomolecular interactions, either specific or non-specific.

SPR sensing on medical diagnostics relies on the fact that $\Delta\theta_{SPR}$ is higher in the case of reactive sera, due to a higher concentration of specific ligands in the samples. As it can be verified from Figure 2A, Au-MPA-CrP sensor platforms have the capacity to distinguish between sera from infected and non-infected individuals (blue and red curves, respectively), and this discrimination becomes better as the serum sample (dashed lines) is diluted more.

Although previous studies have reported that the catalytic domain of TS can be used for the diagnosis of chronic CD,[29] the fact that a TS-based SPR immunosensor cannot distinguish between sera from *T. cruzi* infected patients and control individuals (Figure 2B) can be related to the use of higher serum dilutions in comparison to ELISA, which hinders the possibility of detecting the specific low titer antibodies.[33,34]

The possibility to enhance the detection of the specific anti-*T. cruzi* antibodies in Au-MPA-CrP and Au-MPA-TS sensor platforms by minimizing the impact of non-specific adsorption of serum proteins to the sensor surface still exists. In Figure 3,

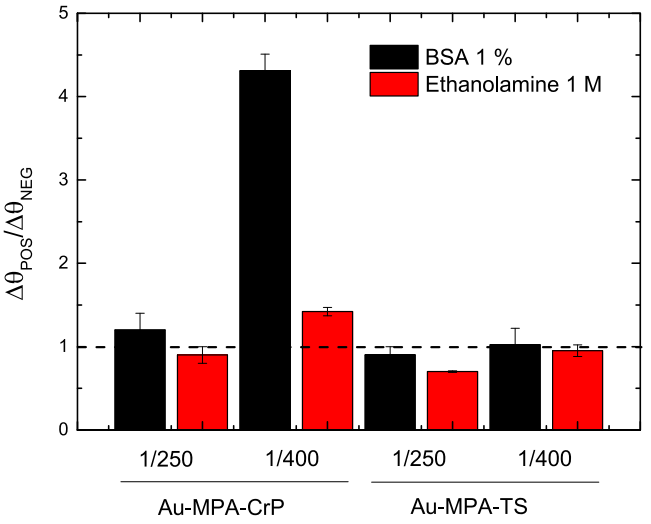

Figure 3. Relative SPR signal ($\Delta\theta_{POS}/\Delta\theta_{NEG}$) from the interactions of Au-MPA-CrP and Au-MPA-TS sensor platforms blocked with 1% BSA or 1 M ethanolamine, pH 8.5, with different serum dilutions. Dashed lines indicate relative SPR signal = 1.

the relative $\Delta\theta_{SPR}$ ($\Delta\theta_{POS}/\Delta\theta_{NEG}$) corresponding to the interaction of positive and negative serum dilutions with Au-MPA-CrP and Au-MPA-TS after the blockage of the non-reactive sites with 1% BSA or 1 M ethanolamine is presented.

Relative SPR signals in the Au-MPA-CrP sensor platform (Figure 3, left) verified that non-specific protein adsorption to the sensor surface can be minimized by using 1% BSA as blocking agent. Compared to ethanolamine (61 Da), BSA (66 463 Da) is an intrinsic surface-active molecule with interfacial adsorption properties, which explains its enhanced ability to block active sites on the sensor chip.[35] It is noteworthy that neither ethanolamine nor BSA as blocking agents can revert the fact that Au-MPA-TS cannot discriminate between *T. cruzi* infected and non-infected individuals, since $\Delta\theta_{POS}/\Delta\theta_{NEG}$ ratios are smaller than one.

Applications in Clinical Chemistry: SPR Immunoassays for the Detection of Anti-T. cruzi Antibodies in Fresh and Heat-Inactivated Human Serum. The performance of Au-MPA-CrP sensor platforms blocked with 1% BSA to detect anti-*T. cruzi* antibodies in clinical samples was evaluated by the injection of heat-inactivated and fresh (non-inactivated) human sera at 1/250, 1/400, and 1/800 dilutions in the SPR device.

Figure 4 shows the $\Delta\theta_{SPR}$ corresponding to the interaction of the sensor surface and heat-inactivated (upper) and fresh

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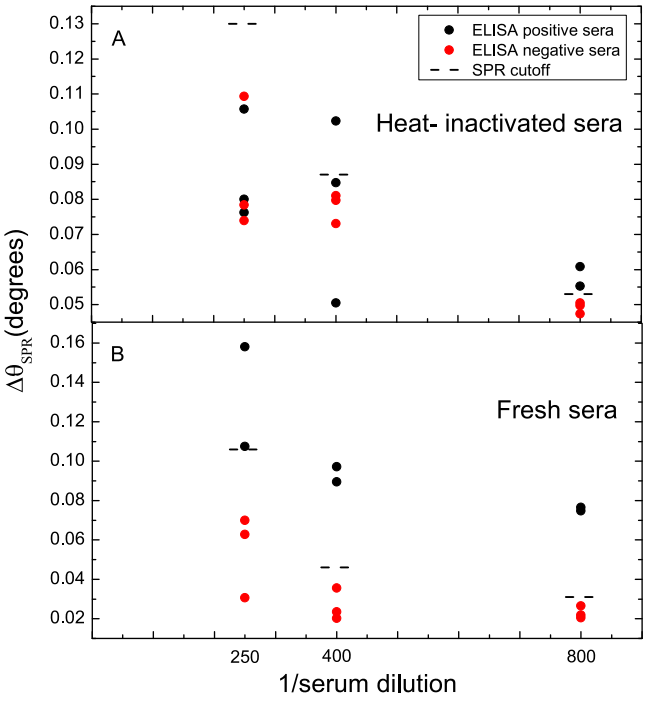

Figure 4. Preliminary evaluation of the performance of the CrP-based SPR immunosensor in the presence of heat-inactivated (upper) and fresh (lower) human serum. The horizontal dotted lines indicate the SPR cutoff values.

(lower) positive and negative serum samples at the different working dilutions.

In order to preliminarily evaluate the potential of the developed CrP-based biosensor to discriminate between samples from infected and non-infected individuals, cutoff values were calculated as means plus three times the standard deviation obtained when performing SPR immunoassays with different dilutions of negative samples. Serum samples rendering $\Delta\theta_{SPR}$ above or below the cutoff are positive or negative for the SPR immunoassay, respectively.

As it can be seen in Figure 4B, CrP-based SPR immunoassays can discriminate between ELISA positive and negative fresh sera samples, and the separation between infected and non-infected groups is more pronounced as the dilution of serum increases, diminishing the risk of serological misinterpretation of the SPR results. When CrP-based SPR immunoassays are performed in heat-inactivated serum (Figure 4A), except in the highest tested dilution, positive ELISA samples appear to be below the SPR cutoff value, resulting in false-negative results as previously reported for heat-pretreated sera in other immunoassays.[18,19] These findings can be related to the production of aggregates of serum proteins resulting from the thermal treatment[36] that interact with the sensor surface, increasing the non-specific signal at the expense of the signal from the specific interactions.

Comparison with ELISA. To assess the validity of the results obtained with the CrP-based immunosensor, it is essential to make a comparison with a routine method for CD diagnosis, i.e., ELISA. Figure 5 shows the values of $\Delta\theta_{SPR}$ obtained after the injection in the SPR device of fresh serum samples at 1/800 dilution from five different individuals, diagnosed as negative (three) and positive (two) by ELISA, IIF, and IHA as a function of the absorbance value obtained by *in-house* ELISA at a 1/400 dilution factor of the samples. The correlation

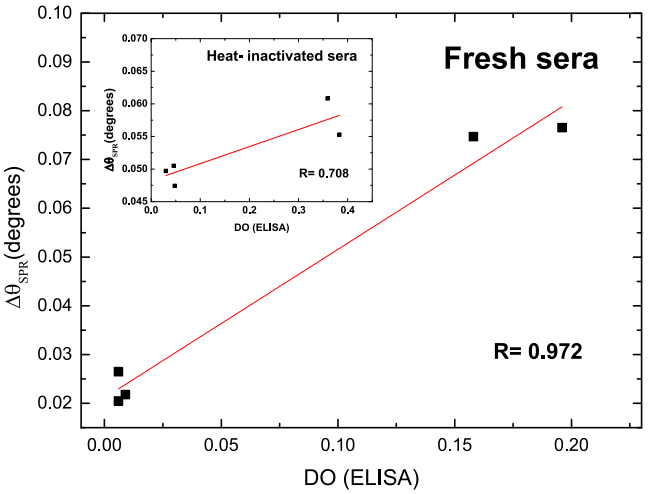

Figure 5. Correlation curve between the obtained results from the analysis of fresh (main graph) and heat-inactivated (inset) human serum samples using the CrP-based SPR immunosensor and ELISA.

coefficient of nearly 1 is indicative of the excellent agreement between the techniques.

SPR results from five heat-pretreated serum samples (three negative and two positive for CD) were plotted against absorbance values obtained by *in-house* ELISA in the same experimental conditions (Figure 4, inset). In this case, a weak correlation (R = 0.708) between SPR and ELISA results is observed, probably due to the different effects of the thermal-induced protein aggregates on both techniques. Whereas the interaction of protein aggregates with the SPR sensor surface directly impacts the SPR signal, the thermal-induced species would not interact with anti-human gamma globulin secondary antibodies responsible for the absorbance signal of *in-house* ELISA.

The fact that the CrP-based SPR biosensor was able to determine greater serum dilutions as positive in comparison to ELISA indicates that the present SPR-based immunosensor can be more sensitive. In fact, SPR experiments suggested that there is greater discrimination for the results obtained with the positive samples compared to those obtained with negative samples when higher serum dilutions are used. This suggests a decrease in non-specific binding in these conditions.

Although SPR and ELISA are sensitive in nearly the same dynamic range,[22] the cost-effectiveness of both methods is an important factor to consider if SPR sensing will be adopted in clinical chemistry. The financial cost of SPR immunoassays mainly depends on the nature of the sensor chip, the number of analyses that can be run with the chip, and the price of the immobilized antigen. In the developed CrP-based SPR immunosensor, the use of bare gold sensor chips, reusable after a simple chemical treatment and purified CrP, yield prices per assay that are comparable to commercial ELISA, opening the possibility to apply this biosensor to remote or resource-limited locations in the point-of-care format.

Conclusions. SPR-based immunoassays carried out with canine sera as a proof-of-concept stage verified that CrP, properly immobilized onto sensor surfaces, allows the detection of anti-*T. cruzi* antibodies. However, recombinant TS retaining the enzymatic activity did not yield specific immunoassays, a finding that can be related to the high dilutions of serum used in the SPR immunoassay that hinders the possibility to detect the specific low titer antibodies.

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The usefulness of the CrP-based SPR immunosensor for the rapid quantification of specific antibodies against *T. cruzi* in fresh clinical samples was demonstrated, and the performance of the biosensor was successfully compared to ELISA. The developed SPR immunoassay, whose price is comparable to commercial ELISA, was able to determine higher serum dilutions as being positive for CD in each case.

In summary, the CrP-based SPR biosensor has been used to detect anti-*T. cruzi* antibodies indicative of CD, and the data collected with this biosensor can be used to perform medical decisions. This early success is encouraging and lays the foundation for the application of the developed SPR biosensor in clinical practices. A greater number of patients will need to be enrolled and larger-scale correlation studies will need to be implemented for the accreditation of this SPR biosensor for clinical tests.

METHODS

Reagents. All used chemicals were of analytical grade. 3-Mercaptopropionic acid (MPA), *N*-hydroxysuccinimide (NHS), ethanolamine hydrochloride, *N*-(3-(dimethylamino)-propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), and bovine serum albumin (BSA, MW = 66.5 kDa) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Phosphate buffered saline (PBS) solution was prepared from potassium chloride; sodium chloride, sodium dihydrogen phosphate, and disodium hydrogen phosphate were obtained from J. T. Baker (Pasadena, Ca, USA). Commercial gold substrates (SPR102-AU) were purchased from BioNavis (Tampere, Finland).

Ligands. TS used in this study was a His-tagged 70 kDa recombinant protein truncated to remove C-terminal repeats but retaining the catalytic N-terminal domain of the enzyme[33] that contains several antigenic determinants located in solvent-exposed areas.[37]

CrP was obtained essentially homogeneous, starting from crude *T. cruzi* epimastigote Y strain extracts, in one step, by affinity chromatography.[38]

Construction of the SPR-Based Immunosensor. *Instrumentation.* Measurements were performed using a SPR Navi 210A (BioNavis) instrument. The setup is equipped with two incident laser wavelengths, 670 and 785 nm, two independent flow channels, and inlet and outlet (waste) tubing. In this work, both of the flow channels were measured in parallel with 785 nm incident light. The temperature of the experiments was kept constant at 22 °C, and the flow rate used for protein immobilization and for monitoring the serum interactions with the immobilized proteins was 10 μL/min. The data presented in this work are averages of three data points.

Formation of Thiol Self-Assembled Monolayers on the Gold Substrate. Single thiol self-assembled monolayers (SAMs) were obtained by overnight incubation of the gold substrates in 1 mM MPA ethanolic solution. The as-prepared surfaces were washed with Milli-Q water and dried with nitrogen before SPR measurements.

Sensor substrates were reused for about ten times after a cleaning treatment in a boiling 1:1:5 solution of 30% ammonia, 30% hydrogen peroxide and water.

Immobilization of T. cruzi Proteins on the Sensor Chip. The immobilization of the proteins to the single MPA SAM was performed by the well-known carbodiimide coupling reaction.[39] The first step was the activation of the MPA-carboxyl groups on the surface with a mixture of 0.010 M EDC and 0.015 M NHS (10 μL/min, 10 min) to give reactive succinimide esters. Subsequently, *T. cruzi* protein solution (120 μg/mL CrP or 15 μg/mL TS) was injected (10 μL/min, 20 min) onto the activated single SAM, and the esters reacted spontaneously with the primary amine groups of the proteins. The immobilization process ended by blocking the remaining succinimide esters using either 1 M ethanolamine, pH 8.5, or 1.0% (w/v) BSA (10 μL/min, 10 min). This *in situ* immobilization was followed in the SPR device by the shift of SPR peak minimum angle (θ_{SPR}). The SPR signal or SPR angle shift ($\Delta\theta_{SPR}$) assigned to the immobilization of the *T. cruzi* protein corresponds to the difference between the value of θ_{SPR} detected before the *T. cruzi* protein injection and the θ_{SPR} value found after it.

Serum Samples. Canine (n = 18) and human (n = 10) serum samples were obtained from individuals either chronically infected or not infected with *T. cruzi*. The diagnosis of canine samples was previously confirmed by ELISA. Human sera included commercial reagents (first International Chagas Standards NIBSC 09/186 2011.2181 and NIBSC 09/188 2011.2181 and negative controls of Wiener Lab Chagatest) and archived clinical samples from Instituto Nacional de Parasitologia (INP), Dr. Mario Fatala Chabén, Buenos Aires, Argentina. Half of the clinical samples were heat-inactivated by heating at 56 °C for 30 min,[40] and half of them were analyzed without pretreatment ("fresh sera").

Serological diagnosis of clinical samples was performed with ELISA, IHA, and IFF with "in-house" antigens (whole cells for IFA and crude cell extracts for IHA and ELISA),[41] compliant with domestic and international rules. When two or three tests were performed simultaneously, the sensitivity ranges from 99.7% to 100%.

SPR Immunoassays between Immobilized T. cruzi Proteins and Anti-T. cruzi Antibodies from Canine Serum. The preliminary evaluation of the immunosensor response and the optimization of the experimental parameters were carried out with canine sera divided into two pools: one consisting of 12 sera from individuals infected with *T. cruzi* (positive pool, POS-) and another consisting of 6 sera from uninfected individuals (negative pool, NEG-). For this purpose, both pools of serum were diluted in 10 mM PBS, pH 7.4. The working dilutions, 1/250 and 1/400, were selected on the basis of a previous report of a related SPR-based immunoassay[32] that recommended a serum dilution of 1/320 as the optimum to discriminate between samples infected and not infected with CD.

Serum dilutions were injected onto the developed sensor platforms, and the interaction between the flowing and the immobilized biomolecules was followed during 10 min in the SPR device by the shift of the SPR angle. After serum injection, a washing step with PBS buffer at pH 7.4 and a regeneration step with 0.5% SDS[31,32] (2 min, 75 μL/min) were performed. At the end of each analysis, the effective $\Delta\theta_{SPR}$ for the samples was obtained through the respective sensograms.

The performance of 1 M ethanolamine, pH 8.5, and 1.0% (w/v) BSA in diminishing the non-specific recognition events between the immobilized proteins of the immunosensor surface and non-related proteins of serum was evaluated in terms of the relative $\Delta\theta_{SPR}$ that characterized the positive serum injection with respect to the $\Delta\theta_{SPR}$ that characterizes the injection of negative serum ($\Delta\theta_{POS}/\Delta\theta_{NEG}$) at different dilutions in PBS.

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SPR Immunoassays with Clinical Samples. The Au-MPA-CrP sensor platform, which yielded the best immunoassays against anti-*T. cruzi* antibodies from canine sera, was tested with clinical serum samples. After CrP immobilization, two blocking steps with 1% BSA (with water as running buffer) and 0.1% BSA (with PBS as running buffer) were carried out in order to diminish non-specific binding.

Heat-inactivated and non-inactivated human sera (at 1/250, 1/400, and 1/800 dilutions) were injected onto the developed sensor platform, and the interaction between the samples and the sensor surface was followed during 10 min in the SPR device by the $\Delta\theta_{SPR}$; after that, a 10 min washing step with PBS buffer at pH 7.4 and a 1 min regeneration step with 0.5% SDS (75 μL/min, 1 min) were performed.

Ethical Approval. The procedure adopted for the manipulation of canine samples was approved by the Animal Use Ethics Committee (CEUA-University of São Paulo, USP; Ribeirão Preto-SP, Brazil), protocol number 08.1.587.53.1

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.9b00269.

> Mean angular SPR shifts used in the estimation of the TS and CrP sensor surface coverage, molecular parameters of the considered close packed array (Table S1), and scheme of the close packed array of globular proteins and unit cell (Scheme S1) (PDF)

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Notes

The authors declare no competing financial interest.

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