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SPR analysis of the interaction between a recombinant protein of unknown function in *Leishmania infantum* immobilised on dendrimers and antibodies of the visceral leishmaniasis: A potential use in immunodiagnosis

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In this work, an SPR immunosensor was developed to elucidate the reaction kinetics between a protein of unknown function in *Leishmania infantum* (hypothetical C1 protein) and specific antibodies of the visceral leishmaniasis (VL). A platform, which is based on layer-by-layer assembly was formed by cysteamine in combination with a fourth-generation poly(amidoamine) dendrimer (PAMAM(G4)) on gold surface for the immobilisation of the protein. This film resulted in amplification of the signal of SPR. Then, a kinetic model based on a bivalent ligation suggested that the reaction between the C1 protein and the anti-C1 antibody occurs in two steps. The value of the equilibrium dissociation constant ($K_{D1} \times K_{D2} = 1.64 \times 10^{-7}$ mol L^{-1}) demonstrated high binding affinity between the biomolecules. Furthermore, low limits of detection (LOD=7.37 nmol L^{-1}) and quantification (LOQ=7.83 nmol L^{-1}) were presented with the proposed SPR immunosensor. Afterwards, the addition of real samples consisting of positive and negative canine sera for VL was accompanied by high sensitivity and selectivity by SPR immunosensor. Therefore, this study quantitatively demonstrated the strong antigenic character of a hypothetical protein and consequently its potential use in the immunodiagnosis of the VL.

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1. Introduction

Leishmania is a protozoan parasite of major medical and veterinary importance (Schoenian et al., 2011). Visceral leishmaniasis (VL), which is also known as kala-azar, is the most serious form of leishmaniasis, in which parasites of Leishmania leave the inoculation site and proliferate in the liver, spleen and bone marrow, resulting in host immunosuppression and, ultimately, death in the absence of treatment (Saporito et al., 2013; Sundar and Chakravarty, 2013). VL is an important emerging parasitic disease in countries around the Mediterranean basin, in the Middle East and in Latin America (Belo et al., 2013). In America, the predominant species is *Leishmania infantum*, which is also known as *Leishmania chagasi* (Alvar et al., 2013; Kaye and Scott, 2011). *L. infantum* antigens are transmitted by the bite of female sandflies, and dogs are the main domestic reservoir (Forestier, 2013; Ready, 2013). Less than 50% of infected dogs present clinical signs of the chronic evolution of viscerocutaneous VL (Forestier, 2013). However, both symptomatic and asymptomatic animals are infected by sandfly vector (Gradoni et al., 1987). Therefore, reliable diagnostic tests for canine visceral leishmaniasis (CVL) are essential for VL surveillance programs and for preventing the unnecessary culling of dogs.

For CVL diagnosis, the methods widely used in clinical routines are the enzyme-linked immunosorbent assay (ELISA) and the indirect immunofluorescence antibody test (IFAT) (Silva et al., 2013; Srividya et al., 2012). However, these methods primarily have limitations regarding low sensitivity in the detection of asymptomatic cases (Michel et al., 2011). Furthermore, these conventional tests employ crude antigen preparations of either whole promastigotes or their soluble extracts, which limit the assay standardisation, reproducibility and present the cross-reactivity with other diseases (Lakhal et al., 2012; Caballero et al., 2007; Frank et al., 2003). Thus, the specificity in CVL serological diagnosis can be improved by the use of recombinant technology over crude antigens (Fernández-Robledo and Vasta, 2010; Terpe, 2006).

As reported by Peacock et al. (2007), *L. infantum* is a diploid cell with approximately 8300 genes, of which 27 are species-specific

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genes. Among these genes, 10 genes have predicted functions, whereas 17 genes are hypothetical because these genes have no known function. Therefore, these hypothetical protein genes are interesting targets for research. Additionally, there is also an incentive to develop immunosensors using new transducers to improve the sensitivity and reproducibility of these assays (Souto et al., 2013).

In this sense, for this work a recombinant protein with unknown function in *L. infantum* (hypothetical C1 protein or C1 antigen), which showed reactive peptides against canine sera infected by *L. infantum* and was not reactive to sera infected with *Trypanosoma cruzi* (Fonseca et al., 2014), was selected as recognition element for the development of an surface plasmon resonance (SPR) immunosensor. The SPR technique was chosen for this study because it permits the evaluation of the antigen–antibody interaction in real time and without the need of chemical or biological markers. This allowed the development of the kinetic model proposed in this work. For this proposal, a layer-by-layer assembled was formed by the adsorption on gold surface of cysteamine followed by the addition of a fourth-generation poly(amidoamine) dendrimer (PAMAM(G4)) for the immobilisation of this C1 antigen (Gibson et al., 2008; Mark et al. 2004). In addition, with the use of a proposed model based on bivalent ligation, it was possible to demonstrate the reaction kinetic and the binding affinity between the C1 antigen and the specific anti-*L. infantum* antibodies.

2. Material and methods

2.1. Reagents and chemicals

The poly(amidoamine) dendrimer (PAMAM, etilenediamine core, generation 4.0 solution 10% (w/w) in methanol); cysteamine (CYS, ≥ 98%); glutaraldehyde (GLU, 25% (v/v) aqueous solution); potassium ferricyanide (III); tween-20 (surfactant P20); and ethanolamine (EA) were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA). Ethylenediamine tetraacetic acid (EDTA); KCl; KOH; ethanol (99%); and monobasic sodium phosphate were obtained from LabSynth LTDA (SP, Brazil). NaCl was purchased from J. T. Baker Chemicals® (Xalostoc, Mexico). The HBS-EP buffer solution at pH 7.4, was prepared using a mixture of 10.0 mmol L^{-1} Hepes at pH 7.4, 150.0 mmol L^{-1} NaCl, 3.0 mmol L^{-1} EDTA at pH 8.0 and 0.005% tween-20. Deionised water was used for solution preparation after purification in a Milli-Q system.

2.2. Apparatus

For the SPR measurements, an Autolab Spirit instrument (Eco Chemie B.V., Ultrech, The Netherlands) was used. Its optical system consisted of a glass prism and a planar gold SPR disc (Eco Chemie B.V., Ultrech, The Netherlands). The equipment has a laser diode with a wavelength fixed at 670 nm. Its operation mode is based on the Kretschmann configuration. A photograph illustrating the equipment, the optical system, and the sensor chip was inserted as Fig. S1 in Supplementary material. Typically, the experiments (23 ± 1 °C) were conducted in HBS-EP buffer solution at pH 7.4 at a flow rate of 48 μL/min. To kinetically evaluate the results the Trace Drawer software, version 1.5 (Oy BioNavis Ltd., Tampere, Finland) was employed.

The apparatus employed for the electrochemistry measurements *are mentioned in* Supplementary material.

2.3. Recombinant C1 antigen of L. infantum, specific IgGs anti-C1, and real samples consisting of positive and negative canine sera for VL

Information about: (1) selection, production and evaluation of antigenicity of the hypothetical C1 protein; (2) production of specific IgGs anti-C1; and (3) real samples employed (positive and negative canine sera for VL) *are given in* Supplementary material.

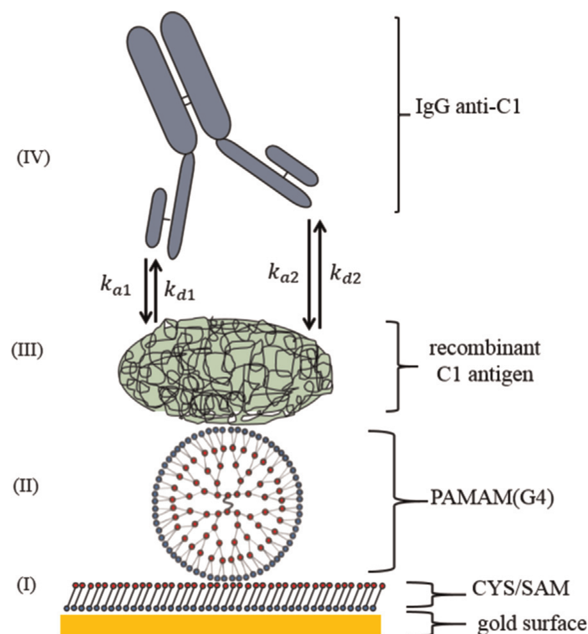

Fig. 1. Scheme illustrating the steps involved in the construction of the immunosensor and its application in the detection of the specific IgGs anti-C1. Formation of the SAM by the addition of CYS on gold surface (I); addition of PAMAM (G4) on previously activated CYS (II); covalent immobilisation of the recombinant C1 antigen on the previously activated PAMAM(G4) (III) and interaction of the specific IgGs anti-C1 with the recombinant C1 antigen (IV).

2.4. SPR immunosensor: construction, characterisation, and evaluation

Each step performed for the SPR immunosensor construction was described in detail below. For easier viewing, a scheme illustrating the steps involved in the construction of the sensor and its application in the detection of specific IgGs anti-C1 was inserted (Fig. 1).

2.4.1. Gold surface modification: formation of CYS–SAM and PAMAM (G4)/SAM

First, the gold substrate was cleaned in piranha solution (1:3 mixture of 30% H_2O_2–H_2SO_4 conc.) for approximately 3–5 min, followed by the immersion of one in acetone (5 min) and then in isopropyl alcohol (5 min). Afterward, the substrates were washed several times with deionized water and dried with a pure $N_{2(g)}$ flow. Then, a SAM was formed by the adsorption of cysteamine (CYS) on the gold surface from an ethanolic solution (2.0 mmol L^{-1}) for 24 h. The formed CYS–SAM/Au was copiously washed with ethanol and dried with a pure $N_{2(g)}$ flow. Then, the terminal amino groups were activated by the addition of an aqueous solution consisting of glutaraldehyde (2.5%) for about 15 min. Next, the PAMAM(G4) 10% (w/w) methanolic solution was spotted onto the CYS–SAM/Au and allowed to incubate overnight for the PAMAM(G4)/SAM/Au formation. In the next step, PAMAM(G4)/SAM/Au was washed three times with methanol and dried with pure $N_{2(g)}$, and the terminal amino groups were activated by introducing glutaraldehyde (2.5%) for approximately 30 min. After the formation and activation of the CYS–SAM/Au and PAMAM(G4)/SAM/Au, each sensor chip was washed several times with water, dried with $N_{2(g)}$ and immediately attached to the prism of the SPR instrument. Then, HBS-EP buffer solution at pH 7.4 was added, and the SPR angle was monitored until the baseline stabilisation.

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