



Synthetic peptides-based SPR biosensor evaluation towards canine Visceral leishmaniasis diagnosis: A simple and effective approach

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ABSTRACT

A vital component of surface plasmon resonance (SPR) biosensors is the recognition element. It generates the sensor selectivity towards the target biomarker and plays an important role in defining the sensor's analytical sensitivity. In serological diagnostics, recombinant proteins (purified antigens) have presented better selectivity compared to soluble crude antigens in SPR immunosensors. However, recombinant DNA technology to obtain proteins is only explored by specialized laboratories, and commercial recombinant proteins still have a high cost. Thus, with the aim of developing a much simpler and lower-cost method, we investigated the prospect of using synthetic peptides as recognition elements for constructing an SPR biosensor. Two synthetic peptides, named PEP13 and PEP16, were tested for the serological diagnosis of Canine Visceral Leishmaniasis (CVL), an endemic neglected disease that affects humans and dogs worldwide. Between the two peptides tested, PEP13 was more sensitive when evaluating its responses against purified antibodies in buffer solution ($\text{LOD} = 1.05 \text{ nmol L}^{-1}$), and it also discriminated the response better when applied in diluted serum samples of infected dogs compared to diluted healthy dogs' samples. For this reason, the PEP13 immunosensor was used to analyze canine serum samples, precisely identifying all the positive ($n = 7$) and negative ($n = 7$) CVL cases ($p = 0.00136$) in less than 12 min. In brief, this study explored promising biostructures through a simple and fast methodology for serological diagnosis, addressing the suitability of synthetic peptides for use in biosensors in the urgent field of neglected diseases.

1. Introduction

In the Americas, Visceral Leishmaniasis (VL) is a zoonotic disease caused by the parasite *Leishmania infantum* mediated mainly by sand flies (*Lutzomyia longipalpis*), with a canine reservoir [1]. It stands out as the most severe form of leishmaniasis, exhibiting a lethality rate of approximately 7 to 10%. This rate may escalate among immunosuppressed individuals, for whom cases can prove fatal if not promptly diagnosed and treated [2–4]. Dogs are the primary reservoir host and can also develop a critical form of the disease (CVL – Canine Visceral Leishmaniasis). Many efforts to prevent dissemination have been made,

and some developing countries have resorted to euthanizing infected animals, causing several societal conflicts [5]. Additionally, most infected individuals remain asymptomatic, playing an important role as parasite carriers, simultaneously making diagnosing it more difficult [6].

To correctly identify a host, most of the time, it is necessary to combine serological, molecular, and cellular tests to obtain a reliable result. Serological strategies such as immunochromatographic strip tests (ICTs), enzyme-linked immunosorbent assay (ELISA), and indirect immunofluorescence antibody test (IFAT) are some of the most popular diagnostic tests, especially due to their high sensibility [1]. However,

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they can sometimes yield false positives due to cross-reactions with other parasitic infections, lacking sensitivity [7]. When considering endemic areas with limited resources, ICTs based on the rk39 antigen detection are a popular choice, offering rapid and cost-effective diagnosis without the necessity of specialized people and equipment [8]. However, they can present lower sensitivity, particularly in early disease stages. Therefore, there is still a need for the development of an ideal diagnostic tool for VL which would present sensitive, specific, user-friendly, portable, and affordable results [9]. In this case, biosensors have been proposed as new alternatives to improve the speed, accuracy, and cost of clinically differentiating healthy from infected dogs [5].

Some studies have attempted to improve VL diagnosis through the development of novel biosensors, exploring different recognition elements [10–13], different immobilization strategies for tethering the recognition elements to the sensor surface [14], comparing transducing techniques [15], developing new surface materials [16,17], exploring the simultaneous detection of multiple analytes [18], and miniaturization approaches for point-of-use detection [19]. Although several studies are approaching different recognition elements, only a few explored peptides for VL diagnosis [12,13]. Peptides present a good perspective when substituting whole proteins due to their great stability, standard synthetic protocol, cost-effectiveness, and tunable physical-chemical properties. However, the use of peptides as recognition elements in optical biosensors applied for VL remains an unexplored area.

Optical-based biosensors such as surface plasmon resonance (SPR) biosensors have grown significantly in recent years as a new diagnostic methodology in clinical analyses. The technique allows real-time measurements with excellent sensitivity and reproducibility, and allows determining important kinetic and thermodynamic experimental parameters and analyzing the binding affinity between different proteins and antibodies of various diseases [20,21]. A key component of an SPR biosensor is the recognition element, which selectively interacts with the target species and, together with the optical transducer, transforms the signal prominent of the biomolecular interaction into a useful analytical signal [22].

The use of synthetic peptides as bioreceptors for the construction of SPR-based biosensors applied to the diagnosis of canine leishmaniasis could contribute significantly to the control of this disease by associating the high sensitivity and speed of response of this optical instrument, which can be, in the future, miniaturized to a portable platform, with the cost-effectiveness and facilities that peptides can offer as bioreceptors, finally offering a rapid and accurate diagnostic alternative [23]. To the best of our knowledge, synthetic peptides have not been explored in SPR biosensors to date. In this context, the present study proposed the construction of an optical device by covalent immobilizing different peptides previously synthesized [24], using SPR as a transduction technique to elucidate the analytical capacity of this synthetic structures to detect *anti-Leishmania* antibodies against real canine samples. Fundamentally, using peptides as bioreceptors could be evaluated and confirmed as promising biomolecules for the construction of future commercial biosensors devices.

2. Materials and methods

2.1. Reagents and chemicals

All the reagents and solutions used in the work are listed below. Aqueous solutions were prepared using purified water from the Milli-Q system ($\rho = 18.2 \text{ M}\Omega$). 3-mercaptopropionic acid (MPA), 11-mercaptopundecanoic acid, N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC), glycine (GLY), and ethanolamine (EA) were obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA). Disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), potassium chloride (KCl), potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$), sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), 30%

hydrogen peroxide (H_2O_2), acetone, isopropanol, and ethanol were acquired from Synth (Diadema, SP, Brazil). Bovine serum albumin (BSA, heat shock fraction V, pH 7.0, 98%) was acquired from Sigma-Aldrich Chemical (St. Louis, MO, USA). The PBS solution (pH 7.4, 0.1 mol L^{-1}) was prepared by equimolar addition of 0.1 mol L^{-1} of KH_2PO_4 , Na_2HPO_4 , and KCl, with pH adjustment by adding NaOH.

The peptides are part of 1 multiepitope protein (PQ20), which has already been proven effective for the serological diagnosis of CVL, constructed according to work developed by Faria and collaborators [25,26]. PQ20 is constituted of 20 peptides that present approximately 95% of B cell epitopes when mapped with BCPreds and ABCPred programs, including PEP13 (sequence KTGKLLGSY) and PEP16 (sequence QSGQFRLGY), the two most reactive epitopes of this chimeric protein [25]. The selected peptides were purchased commercially through the peptide synthesis service of the Laboratory of Immunology and Genomics of Parasites of the Department of Parasitology ICB/UFGM. Specific purified antibodies (anti-PQ20) used in the analytical evaluation and characterization of the biosensor construction were produced by immunizing rabbits with PQ20.

2.2. Apparatus

All the necessary steps for biosensor construction were evaluated via SPR, CV (cyclic voltammetry), EIS (electrochemical impedance spectroscopy), and contact angle. The optical measurements of the SPR angle were obtained to analyze biomolecular interactions using Autolab Springlab equipment (Eco Chemie, The Netherlands). This equipment consists of a prism and a glass disk coated with a nanometric film (50 nm) of gold. It has a cell with a capacity of $300 \mu\text{L}$, and its radiation source is a He-Ne laser with emission at 670 nm. The intensity of the reflected light is measured through a photodiode detector, and the configuration used is based on the attenuated total internal reflection (Kretschmann configuration). The experiments were performed at a temperature of $23 \pm 1^\circ\text{C}$.

Electrochemical characterization measurements of the steps involved in biosensor construction and their detection via impedimetric and voltammetric techniques were performed using a portable Metrohm DropSens STAT-I-400 potentiostat in a conventional electrochemical cell with three electrodes. The reference and counter electrodes were Ag/AgCl/ $\text{KCl}_{(\text{sat})}$ and platinum wire. The working electrode corresponds to a gold electrode of area 3.14 mm^2 . The electrochemical measurements were performed in the presence of the electrochemical probe $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ ($5 \text{ mmol L}^{-1} \text{ K}_3[\text{Fe}(\text{CN})_6]$ solution in PBS). The EIS measurements were obtained by applying a 10 mV alternating voltage (frequency range from 10 mHz to 10 kHz) at the average redox potential, determined as the mean value between the oxidation and reduction processes observed in the CV measurements. The EIS measurements were analyzed using Zview® software (Scribner Associates, North Carolina, United States), obtaining modeling via Randles equivalent circuit, and the model fitting was evaluated through the chi-squared (χ^2) values.

Contact angle measurements were performed on a DataPhysics Instruments GmbH Contact Angle System OCA15 + tensiometer, using a $500 \mu\text{L}$ Hamilton syringe, a 1.65 mm diameter, and a 38.1 mm long needle, completed with ultrapure water at 20°C . The sessile drop method measured the angle from the dripping of $5 \mu\text{L}$ of water on the surface of the modified disks.

The graphs and statistical tests presented in this work were processed using OriginPro software (OriginLab, Massachusetts, United States), with a confidence interval of 95%. In the principal component analysis, the data obtained in this study were processed using Statistica software (StatSoft, Tulsa, United States).

2.3. Biosensor construction

The construction of the biosensors was carried out in six steps: (1) substrate cleaning, (2) functionalization of the metallic surface by

forming SAM_{mix} (mixed self-assembled monolayers), (3) activation of the formed film for covalent anchoring of the recognition unit, (4) immobilization of the peptides, (5) blocking of remaining nonspecific sites, and (6) interaction of the receptors with their corresponding antibodies. Fig. 1 shows a diagram representing the platform used in this study to detect anti-*Leishmania infantum* antibodies.

(1) The 50 nm thick gold surface of the SPR equipment disc (SPR sensor chip) and the gold electrode was cleaned by immersing them in a piranha solution (1:3 mixture of 30% H₂O₂ and concentrated H₂SO₄) for approximately 1 min, followed by sonication in acetone and isopropyl alcohol for 10 min each one. After this process, the substrates were washed 5 times with ultrapure water and dried in a pure nitrogen gas flow. The gold electrode was also subjected to several CV (cyclic voltammetry) cycles in a 0.5 mol L⁻¹ H₂SO₄ solution in the potential range of -0.1 V to 1.5 V and a scanning rate of 50 mV s⁻¹ until the voltammetric response stabilized. Under these conditions, the oxidation potentials of metallic gold to gold oxides and the reduction of gold oxides to elemental gold are 1.27 and 0.90 V, respectively. (2) The functionalization process involved the formation of a SAM_{mix} by immersing the gold substrates in an ethanolic solution of MPA: MUA (0.9:0.1) mmol L⁻¹ for 12 h. After the film formation, the substrate was washed with ethanol and successive washes with water. (3) The formed films were then activated using an aqueous EDC: NHS (10:15) mmol L⁻¹ solution for 20 min, followed by washing with water and PBS buffer solution. (4) The covalent anchoring of peptides PEP13 and PEP16 was achieved by adding a 50 µg mL⁻¹ PBS solution onto the substrate surface for 1 h, followed by successive washing with PBS. (5) a 0.1 mol L⁻¹ glycine solution prepared in PBS was added to the substrate surface for 20 min to block any remaining active sites. This step was also evaluated using other blocking molecules, such as ethanolamine and BSA. (6) The detection step involved the initial addition of PBS to obtain the baseline, followed by the addition of the analysis solution for a determined time. After observing the association event, successive additions of PBS were performed to remove weakly bound species and observe the dissociation behavior. After defining the optimized conditions for the construction of

the immunosensor, the detection step for antibodies anti-*L. infantum* was conducted after obtaining the baseline in PBS for 5 min, adding diluted serum (1:800 dilution factor) for 5 min, with subsequent washing in PBS for 2 min.

2.4. Canine clinical serum samples

The canine sera samples were collected from naturally infected/healthy dogs in Belo Horizonte, Minas Gerais, Brazil. A total of 20 canine blood serum samples were utilized, 10 positives and 10 negatives for CVL in serological and parasitological tests. Two pools were prepared, one utilizing all the positives sera and another the negatives. The canine sera used were tested by IFAT and ELISA simultaneously, and antibody titer determined in IFAT varied from 1:160 to 1:3200.

All experimental procedures involving dogs were carried out in accordance with animal practice as defined by the Internal Ethics Committee in Animal Experimentation (Comissão de Ética no Uso de Animais – CEUA) of Universidade Federal de Minas Gerais (protocol number 198/2014). This protocol followed the guidelines of CONCEA (Conselho Nacional de Controle de Experimentação Animal), the maximum ethics committee in Brazil.

3. Results and discussion

3.1. Biosensor construction and characterization

The construction of the proposed biosensors was evaluated using different techniques, such as SPR, CV, EIS, and contact angle. Initially, the immobilization of the bioreceptors, PEP13 and PEP16, was analyzed in real time via SPR (Fig. 2a). The sensorgrams in Fig. 2A can be divided into three typical regions: the baseline, the bioreceptor binding step, and the washing step. The baseline was obtained by adding PBS pH 7.4 buffer solution, also used to dilute the explored peptides, onto the gold substrate previously functionalized by SAM_{mix} and activated via EDC: NHS. It demonstrates that no significant change in the refractive index is

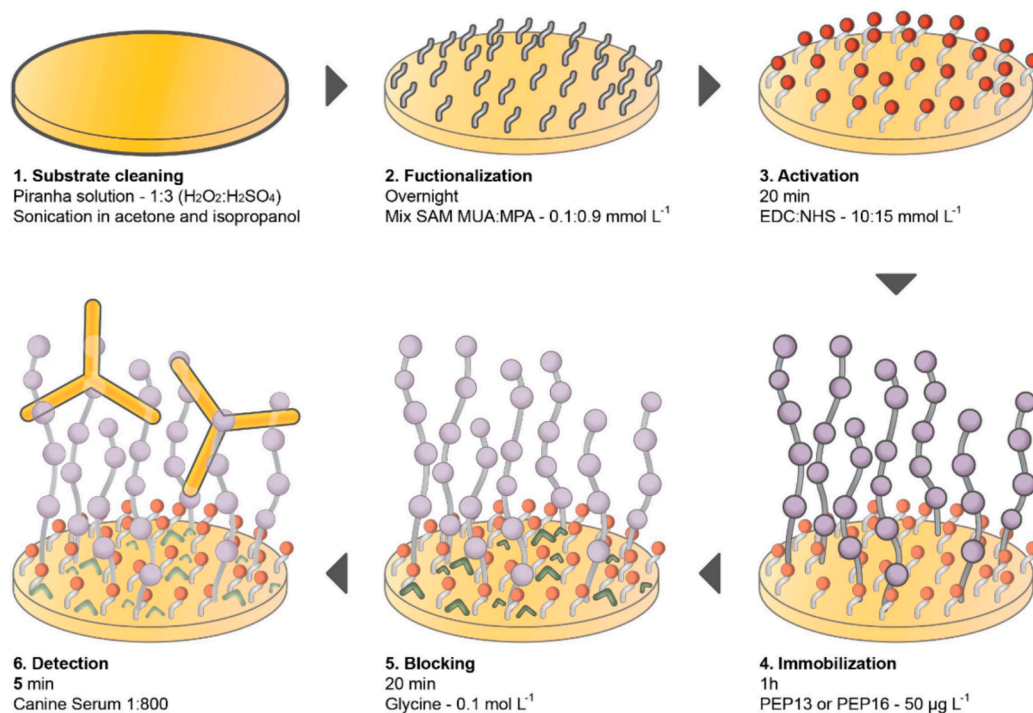


Fig. 1. Scheme of modification steps for biosensors construction. Steps (1) substrate cleaning, (2) functionalization by mix SAM: MUA (0.1 mmol L⁻¹): MPA (0.9 mmol L⁻¹), (3) activation of carboxylic terminal groups by EDC (10 mmol L⁻¹): NHS (15 mmol L⁻¹) aqueous solution, (4) covalent immobilization of the peptides (PEP13 and/or PEP16), (5) blocking of non-reactive groups by glycine (0.1 mmol L⁻¹), (6) detection of LVC antibodies from the standard sample and the canine sera.

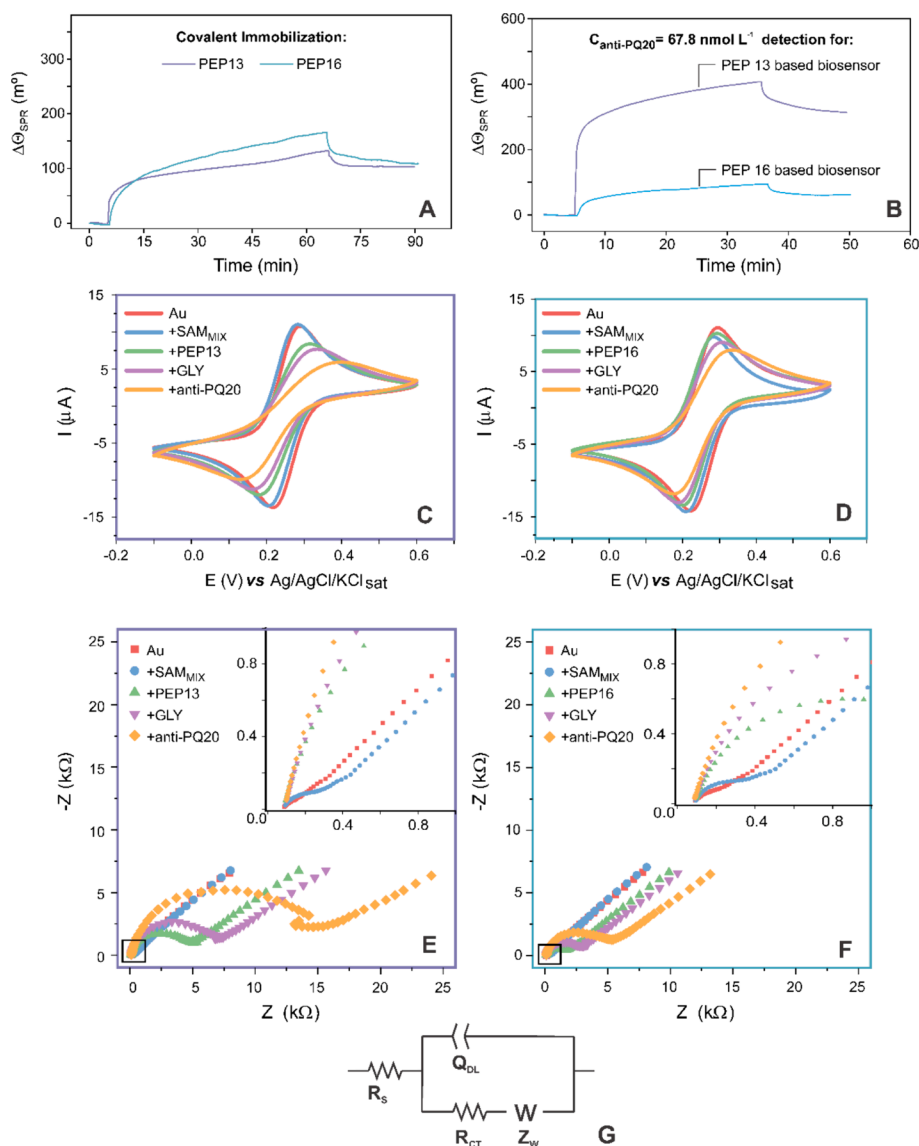


Fig. 2. (A) SPR analysis of the covalent immobilization of PEP13 and PEP16 on the previous SAM_{MIX} functionalized surface and (B) P13 and P16 based biosensor responses to a 67.8 nmol L⁻¹ of an anti-PQ20 solution (purified antibody). Electrochemical characterization using a solution of K₃[Fe(CN)₆] of 5 mmol L⁻¹ via CV and EIS for the steps involved in the construction and detection of each sensor – PEP13-based biosensor (C, E), and PEP16-based biosensor (D, F). The voltammetric measurements were obtained in PBS, scan rate of 20 mV s⁻¹. Corresponded EIS Nyquist diagrams were obtained at $E = 0.25$ V, and (G) respective Randles equivalent circuit was utilized for interpretation of EIS data.

observed over time only in the presence of the buffer, as it does not interact with the dielectric-metal interface. By adding PEP13 and PEP16, which have 14 amino acids each, at the same concentration of 50 μg mL⁻¹, an increase in Θ_{SPR} over time is observed. This indicates a change in the plasmonic wave propagation due to the binding of peptides to the functionalized surface. A pseudo-equilibrium situation was reached for both peptides, indicating a high surface coverage.

After this step, a wash with the same buffer was performed to remove weakly bound species, which was observed for both peptides by the decrease in the angle until reaching a similar effective $\Delta\Theta_{SPR}$, 103.09 m° and 108.48 m° for PEP13 and PEP16, respectively. Furthermore, both showed a small change in the washing step, which may be related to the strong coupling of the peptides to the SAM_{MIX}. Thus, a higher response was observed for PEP13 when evaluating the final variation of $\Delta\Theta_{SPR}$, which could indicate a better anchoring of this peptide on the sensor surface.

Next, the two peptides were tested to determine if there was a response to the target biomarker, the anti-PQ20 antibody. It was

observed the effective variation of Θ_{SPR} for the same concentration of anti-PQ20 antibody (67.8 nmol L⁻¹) for both peptides can be observed in Fig. 2b for each peptide. The binding affinity of the two synthetic peptides to the anti-PQ20 antibody is because the anti-PQ20 antibody was employed in the process of generating the PQ20 chimera protein; where among all peptides generated, the PEP13 and PEP16 sequences presented the strongest binding affinity to anti-PQ20 antibody [24,25].

The steps used to construct biosensors utilizing different bioreceptors were also electrochemically characterized by EIS and CV. In general, the electrochemical behavior of both tested modifications demonstrated, from the addition of the species from each of the necessary stages, an interference mainly in the faradaic processes occurred at the electrode-solution interface. In Fig. 2C and 2D cyclic voltammograms for the surface preparations are presented. A decrease in the total current of the system in all analyzed stages of the surface preparation is observed, indicating a possible increasing electron transfer blocking from redox species in solution ($\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$) to the surface due to all components attached to the surface of the gold electrode. These processes

hinder the access of the $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ probe to the electrode due to their insulating characteristics, which also affect the reversibility of the redox process, highlighted visibly by the more significant separation in the peak potential of the observed oxidation and reduction processes throughout the steps. Before modifying the gold electrode with any species, the voltammetric oxidation and reduction peaks occurred at approximately 0.29 V and 0.22 V, respectively, for both cases (Fig. 2C and 2D). In the SAM_{mix} functionalization stage, the peak potentials remained practically the same, and a very minor decrease in the anodic and cathodic peak currents (I_{pA} and I_{pC} , respectively) was observed. In the subsequent immobilization stage, a more effective separation of approximately 0.02 V in the anodic potential (E_{A}) and 0.04 V in the cathodic potential (E_{C}) was observed for PEP13. While for PEP16, a lower separation was observed, with a displacement of only 0.02 V in the reduction potential. Furthermore, a reduction of 2.42 μA and 0.78 μA of the I_{pA} was concomitantly observed for PEP13 and PEP16, respectively, indicating a greater interface blocking by PEP13, which may potentially represent a more effective immobilization on the functionalized surface. The blocking agent, glycine, also showed respective decreases in the total current and displacement of the potentials of the redox behavior. All these observations confirm a successful preparation of the surface chemistry. Finally, both the sensor surfaces, PEP13 and PEP16, were exposed to a fixed concentration of the target anti-PQ20 (purified antibody). As observed, both sensors responded to the binding of anti-PQ20. However, a greater difference was observed for PEP13, with a $\Delta I_{\text{pA}} = 1.74 \mu\text{A}$, $\Delta E_{\text{A}} = +0.06 \text{ V}$, compared to $\Delta I_{\text{pA}} = 1.07 \mu\text{A}$ and $\Delta E_{\text{A}} = +0.02 \text{ V}$ for PEP16. These results confirm the behavior already observed by SPR, where a more significant response to anti-PQ20 was observed for PEP13.

The electrochemical impedance spectra (Fig. 2E and Fig. 2F) were modulated through Randles equivalent circuit (Fig. 2G), where classic elements of electrical circuits, such as capacitors, resistors, and inductors, can be extrapolated to the general behavior of the system, including the processes that occur at the electrode/solution interface, which facilitates the understanding of the system [27]. The circuit consists of resistance (R_{S}), referring to the sum of each electrical resistance of the electrochemical cell (e.g., gold electrode, electrolyte, cables, contacts, among others), the charge transfer resistance (R_{CT}) – referring to the charge transfer of the faradaic process at the electrode/electrolyte interface, the constant phase element of the electric double layer (Q_{dl}) – corresponding to the formation of the electric double layer at the electrode-solution interface, and the Warburg element (Z_{W}), characteristic of a diffusion-limited reaction. In this case, the constant phase element (Q) is considered since the interface presents a certain deviation “ n ” from ideality compared to a traditional capacitor – where two parallel conductive plates separated by a dielectric material are considered [28]. The calculated parameters for Q_{dl} and R_{CT} are listed in

Table 1
Parameters obtained through fitting EIS data of Fig. 3.

Biosensor Step	$Q_{\text{dl}} / \mu\text{F s}^{n-1}$	ndl	$R_{\text{CT}} / \text{k}\Omega$	χ^2
PEP13				
(1) Bare Au	72.7	0.60	0.222	6.0×10^{-5}
(2) Au-SAM _{mix}	19.4	0.68	0.301	2.4×10^{-4}
(3) Au-SAM _{mix} -PEP	1.56	0.87	4.36	5.9×10^{-4}
(4) Au-SAM _{mix} -PEP-GLY	1.54	0.87	6.56	2.1×10^{-4}
(5) Au-SAM _{mix} -PEP-GLY-AntiPQ20	1.50	0.86	14.0	2.1×10^{-3}
PEP16				
(1) Bare Au	61.7	0.62	0.204	3.81×10^{-5}
(2) Au-SAM _{mix}	9.34	0.78	0.361	1.4×10^{-4}
(3) Au-SAM _{mix} -PEP	2.71	0.85	1.51	1.5×10^{-4}
(4) Au-SAM _{mix} -PEP-GLY	2.94	0.84	2.78	5.4×10^{-4}
(5) Au-SAM _{mix} -PEP-GLY-AntiPQ20	2.59	0.85	4.75	3.7×10^{-4}

Table 1, and the values of R_{S} and Z_{W} did not show any significant change.

Initially, an increase in the semicircle is observed in high-frequency regions in each of the stages studied for both peptides (Fig. 2E and 2F), which would be related to the respective increase in charge transfer resistance of the faradaic process explored in this study, derived from the redox process of the $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ probe. Usually, the stages analyzed by EIS in the modification of electrodes for biosensors mainly impact the electrode/solution interface, thus presenting greater interference in the Q_{dl} and R_{CT} parameters, which was indeed observed for the system studied in this work [28]. Thus, for both studied peptides, an increase in the charge transfer resistance (R_{CT}) was mainly observed, resulting from the insulating characteristic of the components of each of the stages, which, once associated with the gold electrode, hinder the charge transfer referring to the faradaic process of the probe. During the SAM_{mix} functionalization step, the smallest variation in R_{CT} was observed, with an average ΔR_{CT} of $0.12 \pm 0.04 \text{ k}\Omega$ ($n = 3$). This is because a mixed SAM was used, with a higher proportion of the molecule having a shorter carbon chain (MPA), making surface blockage less influential than SAMs formed with larger carbon chain molecules. However, larger variations in the R_{CT} component were observed in subsequent steps, such as during the immobilization, where the larger peptide chains blocked the electrode surface more effectively. The blocking step also showed an increase, with the addition of glycine to avoid nonspecific binding, and in detection due to the association with antibodies, which have a high molecular weight and more effectively block the electrode surface, resulting in a more significant change in R_{CT} . However, PEP13 showed a more substantial surface hindrance, observed by the biggest changes in the R_{CT} values, especially in the immobilization and detection steps. Regarding Q_{dl} , a more significant variation was observed during the functionalization and immobilization steps, unlike the blocking and detection steps, which showed more discreet modifications. The capacitance of the electric double layer is usually related to the electroactive interface's surface area, the dielectric layer's thickness, the medium's permittivity, and the electrolyte's ionic strength [29]. Since the electrolyte remained the same during the measurements, its ionic strength and permittivity were not significantly altered. However, the association of molecules at the electrode-solution interface could be related to an increase in the thickness of the dielectric layer and a decrease in the electroactive surface area, which could be related to the decrease in the Q_{dl} parameter. Comparing the peptides, PEP13 had a greater influence than PEP16 on the Q_{dl} parameter, which is in agreement with the SPR, CV, and electrochemical parameter R_{CT} discussed previously.

A tensiometer carried out another characterization through contact angle measurements, where changes in surface tension due to chemical modifications induced on the surface are observed by changes in the contact angle of the solvent of interest (water) and the surface under study (gold), being a technique widely used in the characterization of functionalized surfaces via SAM [30]. Fig. S1 shows the contact angle images obtained with water on the gold surface at each step of construction of the device. Due to the hydrophobic behavior of the metallic surface, the contact angle is higher (approximately 65°), and it is expected that by adding hydrophilic groups to the interface this angle decrease, which can be observed in the images shown in Fig. S1A, and in the mean plus standard deviation of the measurements performed in different regions of the SPR chip ($n = 5$) shown in Fig. S1B [31].

The biggest significant difference was observed in the functionalization step, demonstrating that the formation of SAM_{MIX} on the gold surface makes it more hydrophilic, possibly due to the presence of terminal $-\text{COOH}$ groups, which is also essential for the chemical environment to be favorable for the natural conformation of the macromolecule, which is more hydrophilic when it is linked to the surface. In the subsequent steps of immobilization, blocking, and anti-PQ20 for PEP13, no significant changes in the contact angle were observed, unlike PEP16, which exhibited a more hydrophilic character after being incorporated

into the surface. This behavior is possibly associated with the nature of the biomolecule, presenting PEP16 more hydrophobic structure when compared to the structure of PEP13 [32].

3.2. Analytical evaluation of the SPR platform against specific leishmaniasis antibody (anti-PQ20)

The analytical response for both biosensors evaluated was obtained at different concentrations of the anti-PQ20 antibody in buffer solutions (Fig. 3). Parameters such as sensitivity and selectivity are essential for an efficient biosensor, being important to detect low levels of the target species while minimizing cross-reactivity with other components [33]. The sensorgrams displayed in Fig. 3A and Fig. 3C show three phases: a baseline with PBS; the association of the anti-PQ20 antibody with the immobilized peptide; and finally, the dissociation of weakly bound species using PBS. For both peptides, the SPR angle varied proportionally with the concentration of anti-PQ20, as shown by the analytical curves in Fig. 3B (PEP13) and Fig. 3D (PEP16). However, PEP13 had a higher variation of the SPR angle, as observed earlier in the surface characterization stages, and could detect anti-PQ20 even smaller concentrations than 33.9 nmol L^{-1} , unlike PEP16. This was confirmed by a sensitivity value of $b = 3.21 \text{ m}^\circ \text{ L nmol}^{-1}$ for PEP13, which was around 7 times higher than for PEP16 ($b = 0.434 \text{ m}^\circ \text{ L nmol}^{-1}$). Furthermore, the linear relationship between $\Delta\theta_{\text{SPR}}$ and the dilution factor for PEP13 was better, with an R^2 value of 0.971 compared to 0.868 for PEP16, which means that a biosensor approach using PEP13 would be more precise for quantitative analysis since the model explains more efficiently the variance of the data. Furthermore, the limit of detection (LOD) and quantification (LOQ) obtained for the PEP13 were 1.05 nmol/L and 3.19 nmol L^{-1} , respectively, and for PEP16, the LOD and LOQ were 9.19 nmol L^{-1} and $27.86 \text{ nmol L}^{-1}$, respectively, which shows that PEP13 can detect significant smaller concentrations of the specific anti-PQ20.

3.3. SPR test in canine blood serum samples

Serial dilution experiments were performed with a blood serum pool

of positive canine sera samples for both peptides, PEP13 (Fig. 4A) and PEP16 (Fig. 4C), to initially assess the performance of both peptides according to their ability to detect antibodies in real samples and optimize the best platform not only over sensitivity but also selectivity. The response of the peptides to blood serum pool of positive canine samples ($n = 10$) showed similar performance when compared to purified anti-PQ20 antibodies response, i.e., PEP13 showed higher sensitivity, but in this case, the linear relationship was compared by the logarithmic function of the dilution factor against the variation of the SPR angle, which can be observed for both peptides (Fig. 4B and Fig. 4D). The logarithmic relationship is often observed in biosensors due to the saturation of active sites for interaction with the target molecule at higher concentrations.

Despite showing a clear difference in sensitivity between the two peptides, specificity against antibodies for LVC also needs to be evaluated. Using blood serum samples for analysis can be challenging, as this complex matrix presents various proteins, antibodies, antigens, electrolytes, hormones, and other substances [34]. In addition to biofouling and the accumulation of biological materials on the sensor surface, nonspecific binding of the recognition unit with other antibodies can also be a problem in choosing the best receptor for the device [35]. For this reason, the response of the biosensors to a blood serum pool of seropositive canine samples ($n = 10$) was compared to a blood serum pool of healthy canine samples ($n = 10$). Effective variations of θ_{SPR} for different dilution factors with PEP13 (Fig. 4E) and PEP16 (Fig. 4F) bioreceptors were compared. In addition to a more sensitive response, it was observed that PEP13 better segregated positive and negative cases, presenting a more specific response in solutions with higher dilution factors. For the PEP13 sensor, from the dilution factor of 1:800 (v:v), no more significant responses related to negative blood serum were observed. The effective variation observed of 125.49 m° would refer only to the interaction of specific anti-PQ20 and PEP13. In this case, PEP16 did not present a wide detection range; the effective value of $\Delta\theta_{\text{SPR}}$ observed without a corresponding negative response was 31.00 m° , lower than that found for PEP13.

In summary, PEP13 provided more promising results in the

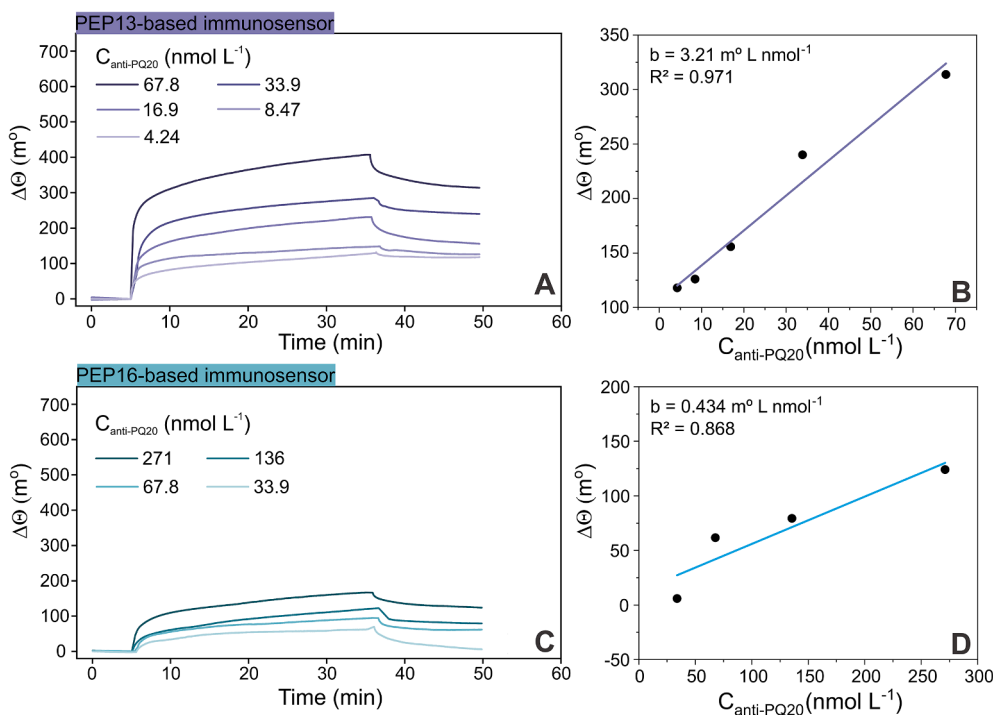


Fig. 3. Sensorgrams ($\Delta\theta_{\text{SPR}}$ vs. time) obtained for the PEP13-based biosensor (A) and PEP16-based biosensor (C) against different concentrations of anti-PQ20, and respective calibration curves for PEP13-based biosensor (B) and PEP16-based biosensor (D).

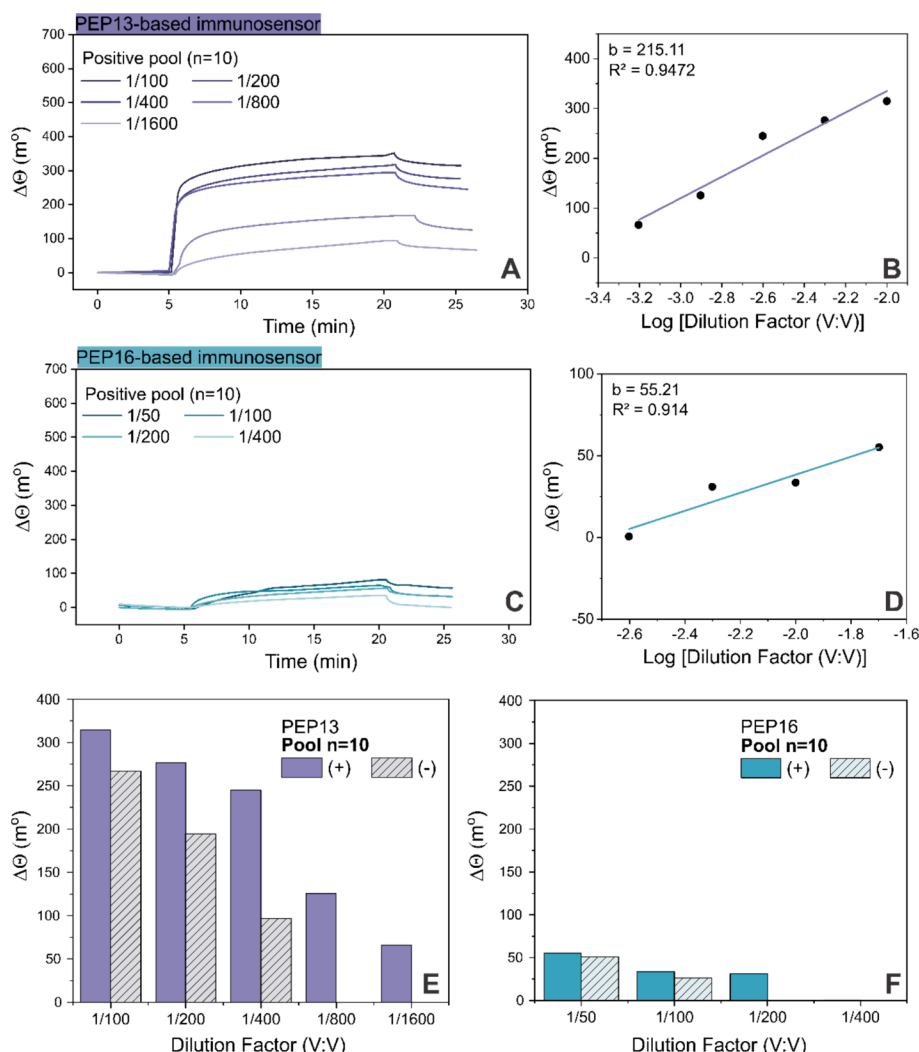


Fig. 4. Sensorgrams ($\Delta\Theta_{SPR}$ vs. time) obtained for PEP13-based biosensor (A) and PEP16-based biosensor (C) against a pool (n = 10) of CVL infected serums in different dilution factors and respective calibration curves for PEP13-based biosensor (B) and PEP16-based biosensor (D). Effective variation of Θ_{SPR} for different dilution factors (v:v) of contaminated (+) and healthy (-) individuals for CVL for the different bioreceptors, PEP13 (E) and PEP16 (F).

characterization stage and, when evaluated analytically, showed better detectability, allowing the detection of lower concentrations of antibodies present in the solution, better detection threshold (e.g., higher effective variation of Θ_{SPR} with increasing analyte concentration), and greater ability to segregate negative from positive responses. For this reason, the biosensor constructed using PEP13 as the bioreceptor was selected for the subsequent stages of optimization of the blocking step, reproducibility testing, and evaluation as a diagnostic tool in individual canine serum samples.

The blocking step is crucial for inhibiting nonspecific active sites on the sensor surface that can significantly affect device selectivity, and three options were tested to optimize it: glycine (GLY) (100 mmol L⁻¹), already used in previous characterization and analytical evaluations, ethanolamine at the same concentration (EA), and bovine serum albumin (BSA) at a concentration of 0.5% (w:v), also commonly used for this function in biosensors.

Using BSA as a blocking agent made detecting both positive and negative samples impossible, indicating a possible steric hindrance between the receptor and the antibody. This is explained by the difference in size between the structures since the BSA used is a protein fraction with a molecular mass of 68 kg mol⁻¹, while peptides have masses of up to 10 kg mol⁻¹. EA and GLY, on the other hand, are comparatively smaller molecules and could therefore be evaluated. It was possible to

observe that for GLY, greater $\Delta\Theta_{SPR}$ were observed for the same dilution factor for both positive and negative samples (Fig. S2A). In addition, a greater difference between the effective variations of positive and negative sera was observed for GLY, with a value of approximately 30% for the positive pool against the negative pool for the dilution factor of 1:200, compared to a 13% difference with EA. Thus, GLY was selected for the blocking step when applying the device in real samples.

Thus, knowing that from the dilution of 1:800, the sensor shows minimized response for negative sera, the reproducibility of the measurement performed for both negative and positive sera at this dilution was evaluated, showing an average $\pm$ standard deviation (SD) response for the pool (+) = 105 $\pm$ 18 m° (n = 5) (Fig. S2). For negative samples, repetitive (n = 3) effective $\Delta\Theta_{SPR}$ responses were not obtained, and therefore, this dilution factor was selected for individual canine serum samples to be tested.

Finally, a stability test was carried out to evaluate the response of the constructed biosensor over time, in which, up to 17 days, the platform did not show a significant change in $\Delta\Theta_{SPR}$ (Table S1), once evaluated through Q-test that all the values could not be considered outliers, retaining 74% of its response after this time. However, a long-term analysis should be performed to elucidate the shelf-life of the platform. Throughout this time, the SPR chip functionalized was kept refrigerated at 4 °C.

3.4. Optimized immunosensor application

Finally, the PEP13 sensor was challenged in seven positive and seven negative canine blood serum samples to demonstrate the feasibility of using the platform as an immunodiagnostic tool for LVC.

In terms of chemometric tools in SPR-based biosensors, it can be noted that their use is still underexplored, and this approach is promising for optimizing the data obtained by the technique. Thus, to improve detection and explore alternative methods of sample classification, we performed a principal component analysis (PCA). PCA is a multivariate statistical technique that reduces the complexity of the original set of variables into a smaller set of independent and unrelated variables while retaining as much information as possible from the original data. Although not a classification technique, PCA is widely used for exploratory observations of trends in data and grouping [36].

The objective of performing PCA was to observe the relationship between the dependent variables and their impact on the separation/selection of positive and negative sample cases. A practical $\Delta\theta_{\text{SPR}}$ value is typically obtained by selecting the angle value after performing the association and dissociation steps within the same period. This is because positive samples containing antibodies that specifically interact with the chosen receptor effectively alter the local refractive index after a similar interaction time with the functionalized surface. However, this approach neglects kinetic aspects that can be observed, especially in the initial stages of the association process, where biomolecular interaction may be favored when species affinity is greater. Since positive samples

are expected to contain specific antibodies, a higher binding affinity is likely to be observed compared to negative samples, which do not contain such antibodies. Additionally, the possibility of reducing analysis time through optimization of data treatment using multivariate analysis can be a significant advantage for biosensors since providing rapid responses is one of the main attractions of these devices over classical diagnostic techniques.

Initially, the raw SPR data from individual samples were used in the PCA, removing the baseline period that does not provide information on the studied biomolecular interaction. Only two principal components were necessary to explain 99.2% of the data variance, with 97.8% attributed to the first principal component (PC1), as can be seen in the score plot (Fig. 5A). A trend of separation between positive and negative samples was observed, mainly concerning PC1, where negative samples were found in the quadrants on the right side (positive value for the x-axis) and positive samples in the quadrants on the left side (negative value for the x-axis), except for sample P6, which showed a similar angle variation to the negative samples.

When evaluating the variables that most influenced the separation trend of the samples by PC1, it was observed that most of them were precisely in the initial association stage and the dissociation stage. For this reason, a second PCA was performed using only the dependent variables of the association stage, considering the first two minutes of this step. Again, with only two principal components, almost all the data variance was justified by the model, with 99.31% for PC1 and 0.58% for PC2, totaling 99.89%. Therefore, from PC1, it was again possible to

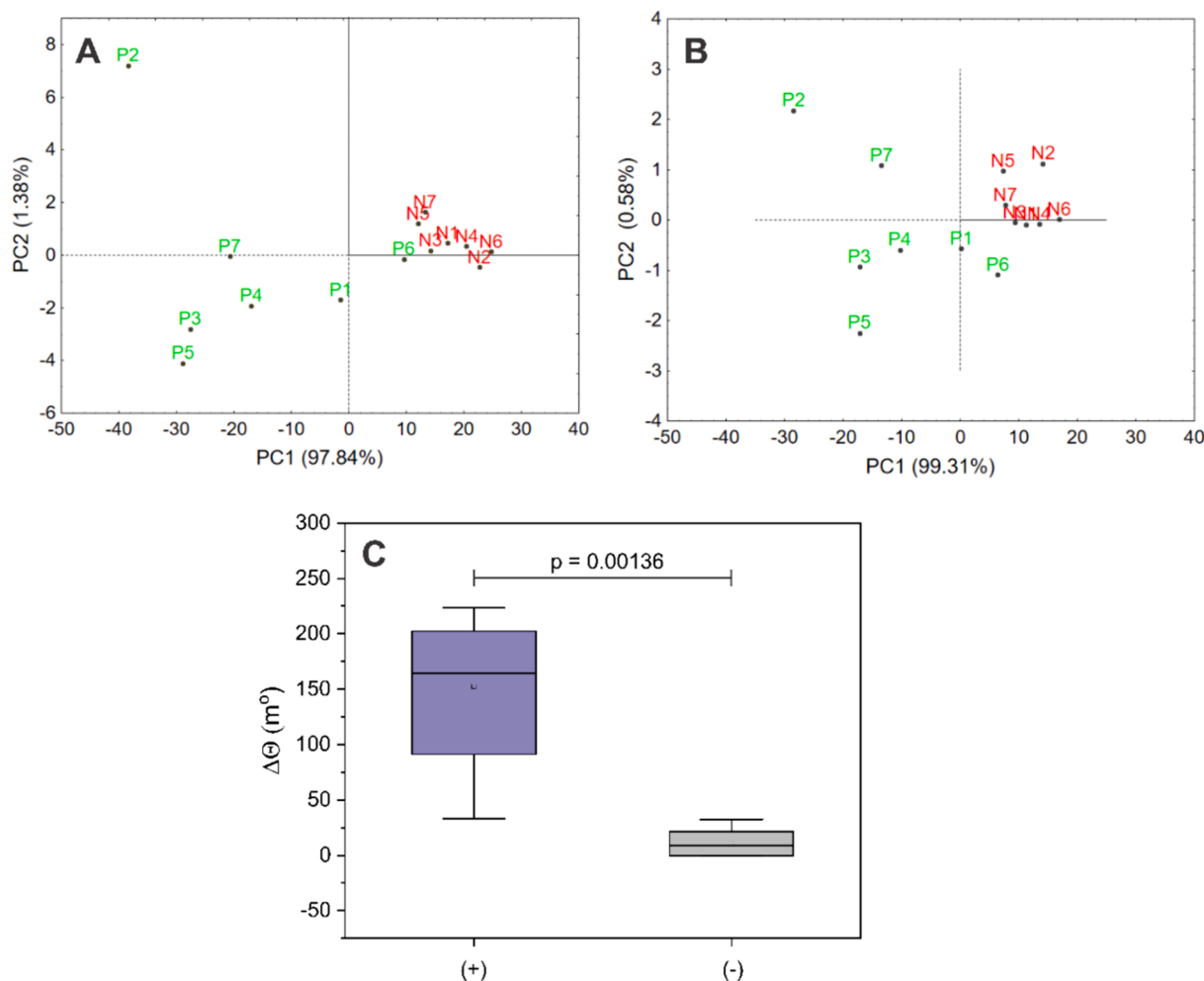


Fig. 5. Scores graph obtained by principal component analysis for raw data (A) and utilizing the first 2 min of the association stage. (B) Positive and negative responses were observed for positive ($n = 7$) and negative ($n = 7$) samples utilizing a cutoff value of 32.624 m° for raw data (C) represented as a box plot.

observe a trend of separation between positive and negative samples (Fig. 5B). That is, with only two minutes of interaction, it was possible to differentiate almost all infected canine serum samples from samples of healthy dogs, except again for sample P6. When analyzing the variables that most influenced the differentiation, all dependent variables showed similar weights, indicating that the separation is mainly due to the magnitude of the observed experimental response.

The observed behavior suggests using even shorter times for detection, which can be associated with future work with multivariate calibration models for sample classification. Moreover, chemometric tools can improve the device response, optimizing its specificity by extracting the most relevant information from the obtained data.

For exploratory analysis of the $\Delta\theta_{\text{SPR}}$ variation among each of the tested samples, positive and negative cases were displayed in boxplots (Fig. 5C), which represent a distribution of data in boxes that represent the 50% quartile range, meaning where 50% of the samples are in terms of effective θ_{SPR} variation. In addition, the median, mean, and standard deviation values can also be visualized. As expected, due to positive cases' dependence on the patient's immune response, a higher variance was observed for positive samples and a higher mean response, with an average response 15X higher for samples from dogs infected with LVC compared to negative cases. Additionally, the groups showed a significant statistical difference, with $p < 0.05$ (Fig. 5C).

Based on the analysis of both positive and negative sera, a cut-off value of 32.62 m° was established. This threshold allowed the platform to accurately identify all individual serum samples. However, it's important to emphasize the necessity of testing a larger sample size for diagnostic test validation, encompassing asymptomatic cases as well. This broader testing scope is crucial for the methodology to be truly representative of the tested population. Furthermore, it will help evaluate whether the proposed approach can effectively address the challenge commonly encountered in serological diagnosis for CVL: the identification of asymptomatic cases, which frequently exhibit low reactivity.

Another platform based on peptides organized into polyamide-6/chitosan nanofibers for the detection of visceral leishmaniasis antibodies was studied by Liberato et al. [16]. The work used electrospinning to produce nanofibers that were functionalized with synthetic peptides specific to VL. The peptide used was derived from the promastigote surface antigen, which is a soluble antigen responsible for activating the cellular immune system from the initial stages of infection. The impedimetric biosensor had a detection limit of 0.2 pg mL^{-1} . Additionally, the sensor was exposed to positive and negative LV serum samples, allowing for effective differentiation. Braz and collaborators evaluated two solid-binding peptides as bioreceptors in another electrochemical platform based on graphene-based disposable electrodes, which were able to differentiate positive and negative serums for VL [37]. Electrochemical methods may offer higher sensitivity, a crucial factor for identifying the initial stages of a disease. However, the requirement for electrochemical redox probes to evaluate faradaic processes at the interface can be a disadvantage, especially when considering the complexity of the sample preparation process.

Some studies have applied peptides for VL diagnosis, including ELISA assays [24,38], photoelectrochemical biosensors [13], and rapid diagnostic tests [39]. Peptides offer a promising avenue for enhancing antibody detection by targeting more specific epitopes, thereby mitigating undesired reactions. Their simplified synthesis, independent of living organisms, not only reduces costs but also improves reproducibility in production [40].

This study exploring peptides in SPR biosensors stands out in comparison with the exemplified techniques for its simplicity, rapid response, satisfactory sensitivity and selectivity, and potential for multiplexing and miniaturization [41]. These attributes are crucial for applications such as CVL diagnosis, which faces socioeconomic barriers due to resource-intensive strategies.

4. Conclusion

The proposed methodology highlights the use of synthetic peptides as bio-receptors in SPR technology since these molecules stand out for their high binding affinity with target antibodies and their greater ease of large-scale production and stability. These advantages are interesting for their commercial use in place of proteins.

Two synthetic peptides, PEP13 and PEP16, were evaluated as recognition units in constructing an optical biosensor based on the SPR technique for use in Canine Visceral Leishmaniasis immunodiagnosis. The results demonstrated that PEP13 had a more attractive analytical performance when evaluating parameters such as sensitivity, specificity, detectability, and working range despite both promising results, allowing for efficient separation of positive and negative sera. Additionally, a relatively quick diagnosis was achieved, with a final response obtained in 10 min of analysis, which can be further optimized when associated with chemometric techniques since PCA showed that in the first 2 min of the dissociation step, a separation trend of the evaluated real samples was already observed.

Finally, developing biosensors for diagnosing Canine Visceral Leishmaniasis and other neglected diseases is extremely important for public health. The use of synthetic peptides in these assays can considerably reduce their costs, and the feasibility of SPR biosensors can allow access to a quality test that does not depend on infrastructure or high financial resources.

CRedit authorship contribution statement

Jaqueline Volpe: Writing – original draft, Methodology, Investigation, Conceptualization. **Gabriela P. Parchen:** Methodology, Conceptualization. **Floriatan S. Costa:** Writing – original draft, Methodology, Conceptualization. **Adriano de Souza Silva:** Visualization, Conceptualization. **Hélida M. Andrade:** Writing – original draft. **Clarice D. B. Amaral:** Writing – original draft, Conceptualization. **Saimon M. Silva:** Writing – original draft, Conceptualization. **Lauro T. Kubota:** Visualization, Conceptualization. **Dênio E. P. Souto:** Writing – review & editing, Supervision, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.microc.2024.110844>.

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