Design and characterization of high-affinity synthetic peptides as bioreceptors for diagnosis of Cutaneous Leishmaniasis

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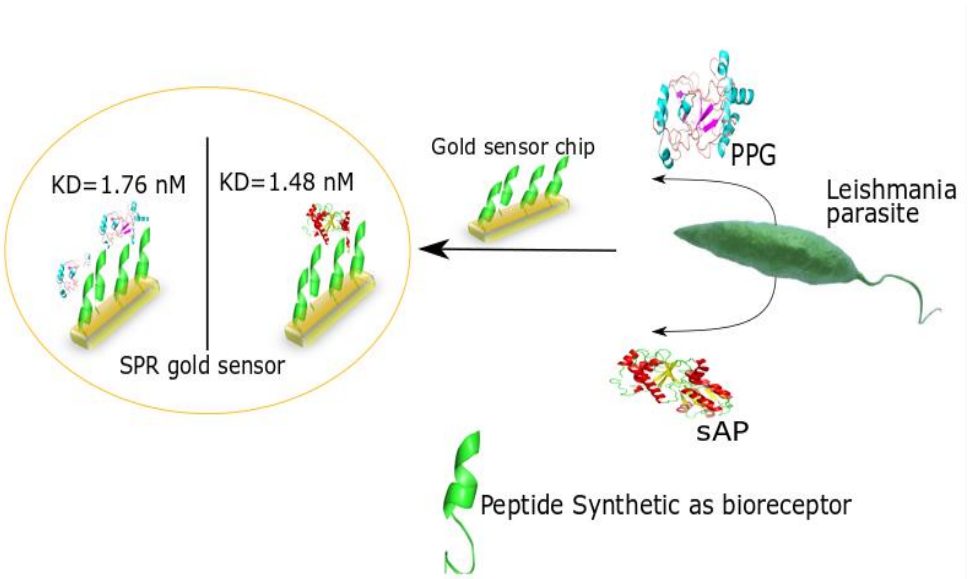
Graphical Abstract



Abstract

Cutaneous leishmaniasis (CL) is one of the illness caused by Leishmania parasites infection, which can be asymptomatic or severe according to the Leishmania strain infecting. CL is commonly diagnosed by directly detecting the parasites or their DNA in tissue samples. New diagnostic methodologies target specific proteins (biomarkers) secreted by the parasite during its infection process. However, specific bioreceptors for the *in vivo* or *in vitro* detection of these novel biomarkers are rather limited in terms of sensitivity and specificity. For this reason, we here introduce three novel peptides as bioreceptors for the highly sensitive and selective identification of acid phosphatase (sAP) and proteophosphoglycan (PPG), which have a crucial role in leishmaniasis infection. These high-affinity peptides have been designed from the conservative domains of the lectins family, holding the ability to interact with the biological target and produce the same effect that the original protein. The synthetic peptides have been characterized and the affinity and kinetic constants for their interaction with the targets (sAP and PPG) have been determined by a Surface Plasmon Resonance biosensor. Values obtained for K_D are in the nanomolar range, which is comparable to high-affinity antibodies, with the additional advantage of a high biochemical stability and simpler production. Pep2854 exhibited a higher affinity for sAP (K_D =1.47 nM) while Pep2856 had a good affinity for PPG (K_D 1.76 nM). This study evidences that these peptidomimetics represent a novel alternative tool to the use of high molecular weight proteins for biorecognition in the diagnostic test and biosensor devices for CL.

Keywords: Cutaneous leishmaniasis; high affinity peptides; lectins; protephosphoglycans; acid phosphatase

Introduction

Leishmaniasis, caused by Leishmania parasites, is one of the infectious diseases with the highest incidence in the tropical regions of South America, Africa, the Mediterranean region, and Central Asia, affecting nearly 100 countries. According to the World Health Organization (WHO), there are one million cases of infection each year, including asymptomatic cases which are not reported [1,2]. Leishmaniasis affects the most vulnerable population in the world, usually associated with inadequate nutrition, immunosuppression, and deficiency in the public health system. There are three clinical manifestations of leishmaniasis infection: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML), and visceral leishmaniasis (VL), known as kala-azar [3]. CL is the most frequent form of the disease; it causes skin lesions that fortunately can heal without treatment in many cases [4]. ML occurs when the parasite spreads into the nose, mouth, and throat, and it produces severe ulcers, destroying the mucous membranes. It can be cured, but the symptoms might not be detected until years after infection and, if not treated, it can evolve in serious complications [5]. VL is the most lethal form of the disease; it damages internal organs like spleen or liver, and it affects the bone marrow and the immune system, which results fatal for the patient and death may occur due to complications associated with secondary infections [6].

To date, there is no vaccine available against leishmaniasis. However, a recent study predicted 3D structure of a vaccine candidate against VL caused by *Leishamania donovani* based on VC1/VC2 modelled using a molecular dynamics coupled TLR-2 and TLR4 receptors [7]. Therefore an accurate and early diagnosis of the disease is crucial for the timely administration of treatment and the reduction of the infectious burden [8,9]. Importantly, due to the geographic and sociologic distribution of leishmaniasis incidence, it is essential to develop new diagnostic tests that can be implemented as point of care (POC) devices and applied for routine screening analysis [10,11]. The broad spectrum of clinical manifestations of CL makes its timely diagnosis complicated. Currently, standard methods for the diagnosis of CL include microscopy and microbiological culture of the parasite, which require centralized laboratories, highly specialized personnel, and up to several days for providing conclusive results [12]. The detection of CL via blood serological tests shows a very low sensitivity due to a deficient humoral response by the immune system [13]. This is because the immunological effect in humans is induced by the parasite amastigote form (reproductive phase) and not by the promastigote (infective phase), which infects the macrophage cells immediately after contagion [14]. Clinical assays targeting gene sequences of the parasite using the polymerase chain reaction (PCR) technique have also been developed, as well as immunochemical tests targeting antigens of the cell membrane, such as Kalazar Detect and IT-LEISH for VL, based on recombinant Leishmania protein K39, and Montenegro skin test (MTS) for CL [15,16]. Other candidates for the detection of leishmaniasis are the membrane glycoproteins gp63, gp70 and gp72 which are specific for the Leishmania genus, and have improved the sensitivity and specificity of ELISA tests [17].

However, these are prone to cross-reactions with other diseases caused by trypanosomes family members. The limitations of the conventional diagnostic methods are driving research efforts towards the finding of direct biomarkers and potential targets to increase the sensitivity of the diagnosis [18]. Recently, secreted proteins are considered an untapped source of possible antigens and are being exploited using combinations of bioinformatic

and immunoproteomic approaches [19,20]. Our focus is directed to the glycoproteins secreted during the infectious process by Leishmania parasite, which are promising biomarkers for the detection of CL. Among them, the acid phosphatases (sAP) and protephosphoglycans (PPG) are secreted by the parasite mostly during the infective phase associated to the promastigote form; therefore, they can play a critical role as clinical targets for both diagnosis and vaccine development [21].

The sAP are complex enzymes, N-carbohydrate and predominant O-linked phosphorylated, with molecular weight between 90-130 kDa, that are considered a virulence factor [22]. The sAP inhibits the production of superoxide anions by neutrophils and macrophages, closely related to the survival of the parasite inside the host cell. The protephosphoglycans (PPG), with a molecular weight around 200 kDa, are involved in the mammalian infection and may contribute to the binding of Leishmania to the host cell [23].

Nowadays, it is well known that the interaction of mannose-binding lectin with the specific carbohydrates of glycoconjugates on the cell surface of Leishmania acts as an alternative pathway in the complement activation mechanism antibody-independent response [24,25]. In this sense, lectins can recognize specific glycan arrangements in the PPG molecules [26]. The aim of this work is to design, synthesize, and characterize a new peptidomimetic-based galactolectins with enhanced affinities and robustness for both sAP and PPG, to be used for the highly sensitive detection of Leishmaniasis. [28,29].

Based on the rational bioinformatic design and theoretical modelling of the interaction, we have obtained three peptide sequences specifically targeting small regions (10-15 amino acid residues) of the lectin domains that can keep the structure-activity relationship [27]. Peptides were synthesized using Fmoc solid-phase peptide chemistry. Synthetic peptides have been fully characterized to determine the binding affinities and kinetic parameters for the interaction with Leishmania PPG and sAP proteins by Surface Plasmon Resonance (SPR) biosensor analysis [28]. Our findings demonstrate the valuable potential of the new synthetic peptides as bioreceptors for the direct assay of leishmaniasis biomarkers in diagnostic tests. The higher affinity and stability of our receptors might greatly enhance the analytical performance of conventional clinical assays or innovative diagnostic devices, such as point-of-care biosensors.

Materials and Methods

General Chemistry

All common reagents and materials were obtained from a commercial source as Sigma Aldrich and MERCK and used without further purification unless otherwise indicated. Glassware was oven-dried before use and the solutions prepared in deionized water (Resistivity 18.2 MΩ).

Bioinformatic peptide design

The protein sequences of secreted acid phosphatase and proteophosphoglycans reported by *Leishmania ssp* parasites were selected on Uniprot Data Bank (Available on: www.uniprot.org/). About twelve sequences of sAP and four sequences of PPG with up to 60% identity analysed and aligned using the both, BLAST and Clustal Omega tools (Available on Uniprot) for finding a conservative domain in an independent bioinformatic analysis are reported in the Supplementary Information **Figure. S1a-S1b**. Subsequently, the native models for both sAP and PPG conservative regions were obtained from the GalaxyWEB web server, which allowed making the prediction, refinement, and optimization of the protein structure based on protein-template by rescoring HHsearch results. Then a group of 15 sequences of glycoconjugate-recognized lectins were selected from Protein Data Bank and ten fragment peptides between 14-18 amino acid residues of different motif regions of the proteins which were extracted from Protein Data Bank (https://www.rcsb.org/) with ID codes: 1CIW; ICQ9; ICR7; IQF3; 1RIR; 2DHI; 2DV9; 2DVA;2DVB;2DVD; 2DVF; 2DVG; 2DVP; 2TEP; 1BZW. The 2D conformation of each peptide was modelled and visualized using the PEPFOLD server and PyMol v2.3 respectively. The experimental data suggest that three peptides sequences described as Pep2854, Pep2855, and Pep2856 adopted a conformational space-optimized based on high interaction similarity with the targets.

Theoretical modelling of peptide-glycoproteins interactions

CABS-Dock server (http://biocomp.chem.uw.edu.pl/CABSdock) was used to investigate the protein-peptide interaction. For the performance of the dynamic simulation, the algorithms found the binding site with full spatial flexibility of peptide and decreased the fluctuation on the protein backbone without an a priori knowledge of the binding site [29]. To evaluate the results, the RMSD value was used, which is defined as the root mean square deviation of the peptide-protein interaction. For selecting the better models, the contact maps were calculated with a cut-off distance value of 4.5 Å for each peptide-glycoconjugate complex (Supporting Information **Figure. S3a-S3b**).

Synthesis and purification of peptides

Pep2854 (HYSRTENMGTSNAC), Pep2855 (KTFLKICKRPYVPSEP) and Pep2856 (INKQGDVTVLSNGNTQLT) were synthesized by solid-phase peptide synthesis following the Fmoc methodology. Briefly, peptides building blocks were loaded on of Rink amide 4MBHA resin (0.55 mmol/g substitution;100 mg per *tea-bag*) followed by chain linkage via the coupling of Fmoc-protected-L-amino acids between the C-terminal residue and the resin. The amino acids were coupled using a mixture of the L-amino acid, O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) or , oxime and N-Ethyldiisopropylamine (DIEA) in this ration 5:5:5:7.5 respectively for over 3 hours. The Fmoc protecting groups were removed with piperidine (20% v/v in 0.01% Tween 20 solution). All coupling reactions were carried out in dimethylformamide. The peptides were cleaved and the lateral chain protecting groups were removed using a cocktail containing trifluoroacetic acid (TFA), triisopropylsilane (TIS) and water (ratio 95:2.5:2.5). Lastly, the peptides were precipitated with cold diethyl ether, centrifuged thrice and the pellet was dissolved in water before lyophilization. The crude peptides were purified by RP-HPLC on a Zorbax C-18

preparative column (7.0 µm; 21.5 x 250 mm) using a gradient from 30% to 70% of acetonitrile (ACN) (Mobil phase A: H_2O, 0.001% TFA and B: ACN, 0.001% TFA.

Mass spectrometry analysis

The fractions collected from RP-HPLC were analyzed by MALDI-TOF spectrometry in an UltrafleXtreme Bruker Daltonics mass spectrometer using a dried-droplet method using an α-Cyano-4-hydroxycinnamic acid saturated solution (0.001% TFA in ACN/ H_2O) as matrix substance for MALDI.

Circular dichroism analysis

2D peptide structures were analyzed by circular dichroism in a Jasco J-815 spectropolarimeter with an acquisition speed of 20 nm/min in a spectral region of 190-260 nm.

Surface Plasmon Resonance biosensor

SPR experiments were performed at room temperature (25°C) on a home-made SPR biosensor. The sensor platform is based on the Kretschmann configuration that allowed the monitoring of the spectrum of the reflected light at 70° of incident angle on a 50 nm layer of the gold sensor chip, previously biofunctionalized with each peptide. The biosensor surface is clamped in a flow cell and is irradiated with a halogen lamp (HL-2000, Ocean Optics USA) in transverse-electric polarization mode; the light passes through a trapezoidal prism for plasmonic excitation. Then, the reflected light is collected by an optical fiber-coupled to a CCD spectrometer (Ocean Optics-Jazz Module, USA). The microfluid delivery system includes a syringe pump (New Era, NE1000, USA), connected to the flow cell. Samples of sAP and PPG were manually injected using an injection valve (IDEZ Health and Science, V-451, USA). Thereby, the biomolecular interactions that take place on the sensor surface can be detected by the changes in the refraction index and the displacement of the plasmon wavelength ($\Delta\lambda_{SPR}$). Data are presented as a sensorgram where the $\Delta\lambda_{SPR}$ is evaluated in real-time.

Sensor surface biofunctionalization

The sensor chip is based on an ultra-thin gold layer (50 nm thickness) deposited on a glass slide previously covered with titanium (2.0 nm thickness). The sensor chips were washed with acetone, isopropanol, and Milli-Q water by immersing at boiling temperature. After that, they were dried with N_2 and placed in an ozone cleaner (BioForce Nanoscience, USA) for 40 min. A self-assembled monolayer (SAM) was carried out ex-situ using a mixture of functional alkanethiols, mercaptohexadecanoic acid (MHDA) and mercaptoundecanol (MUOH) in a 1:1 ratio and total concentration of 250 µM in ethanol, where the sensor chips were incubated overnight. Then, the nanoplasmonic sensors were rinsed with ethanol and dried with N_2. The carboxylic group activation was performed via carbodiimide esters formation using a mixture of 1-etil-3-(3-dimethylaminopropil)carbodiimide (EDC) and N-hydroxysuccinimide ester (NHS) (0.2 M/0.05 M) solution in 2-(N-morpholino)ethanesulfonic

acid (MES) buffer (100 mM; pH 6.8) for 30 min. Afterward, each peptide (Pep2854, Pep2855, and Pep2856) was covalently immobilized on a gold sensor chip using a 50 µg mL^{-1} peptide solution in MES buffer (50 mM; pH 7.0) by incubation overnight to ensure a high density of bioreceptor on the sensor chip surface. Finally, ethanolamine (1M; pH 8.5) was added to deactivate the unreactive carboxylic group. The sensor chips were rinsed with Milli-Q water and dried with N_2.

Binding interaction analysis on sensor chips

The dilution series of targets were injected (sAP and PPG) in separate experiments for each peptide without exceeding the saturation concentration of the analyte onto the sensor chip surface. In the case of sAP, the range of concentrations injected was from 5.2 to 104 nM in 10 mM MES buffer pH 6.5. Moreover, the PPG was injected in concentrations between 3.0 and 58 nM prepared in 10 mM acetate buffer pH 4.5. The running buffer for each assay was the same as for the sample to avoid a bulk refractive index effect, and the flow rate was 30 µL/min applied for 15 min. The regeneration step was carried out so that all peptides were able to dissociate entirely using 5.0 mM of NaOH at a flow rate of 30 µL/min for 2 min. The sensorgrams acquired were fitted to a one-site binding interaction model using GraphPrism software (Version 9.0). This model assumed that the formation of the target-ligand complex is not limited by mass transport because the SPR biosensor is capable of keeping a constant target concentration in a continuous flow. Therefore, in the kinetic analysis, the constants of association (k_{on}) and dissociation (k_{off}) were calculated from the plot of $\Delta\lambda_{SPR}$ versus concentration[30]. Consequently, the equilibrium dissociation constant (K_D), known as affinity, was determined from the relation $K_D = k_{off}/k_{on}$. Additionally, the Chi^2 value was reported as a parameter indicative of the fitting confidence.

For the determination of SPR sensitivity we used buffer PBS serial solutions prepared in MilliQ water; then refraction index of each PBS solution was measured in a Refractometer and were injected in order of less to major concentration. Then $\Delta\lambda$ was determined from the LSPR vs Real time graphical as shown in the **Figure. S4** of Supporting Information.

Results and analysis

Design and synthesis of specific PPG and sAP bioreceptor peptides

We have developed three new peptidomimetic molecules for the sensitive and selective detection of PPC and sAP proteins, secreted by Leishmania parasite during the infection process. The rational design of peptides was based on the conservative domains of lectins specific to cell-surface carbohydrates as the Gal-Man-(PO4) type and on the analysis of the active sites which were previously identified and reported on the Protein Data Bank (PDB). The finding conserved domains in sAP and PPG are able to predict the interaction between peptides and these biomolecules which will mostly take place in these regions increasing the probability that the peptides designed can recognize any sAP or PPG secreted by a broad range number of *Leishmania* species. Therefore, from the Clustal Omega results of the sAP secreted by Leishmania reported in Uniprot, we selected the region

corresponding to MVQVVHRHGARSALNDNTTEICGTLYPC (Code Job Uniprot: /www.uniprot.org/align/A20190117216DA2B77BFBD2E6699CA9B6D1C41EB202DE21L) and for PPG we choose the region corresponding to 1030-1450 of the proteophosphoglycan with A4HM87 as more conservative region by the only four sequences reported (Code Job Uniprot:www.uniprot.org/align/A20190918674680338 1A1F0E0DB47453E0216320D05A710T.aln). The PDB codes, Clustal Omega and the BLAST results are included in the Supporting Information in the **Figure. S1a-S1b**.

The peptides were synthesized and purified using Sepack C-18 columns, and the 2D structure was analysed by circular dichroism as shown in **Fig. 1**. The 2D structure of a lectin reported in the Protein Data Bank with PDB code 2DHI. These proteins family are characterized by showing mostly both beta-folded and an α-helix motifs structure. In addition, the peptides retain the 2D structure, a very important observation considering that peptides having less than 20 amino acids are crucial in the active design of functional peptides that can access to a specific biological target. The Pep2854 and Pep2855 exhibited a strong tendency to an α-helix structure as a result of the presence of amino acids such as His, Cys, Glu, Asp and Ala, while Pep2856 showed ß-sheet profile, favoured by the presence of Gly, Val, Ile, and Leu residues (Fig. 2b-2c). Our experimental data support the results of the theoretical folding of the most likely conformation peptides performed on the PEPFOLD server.

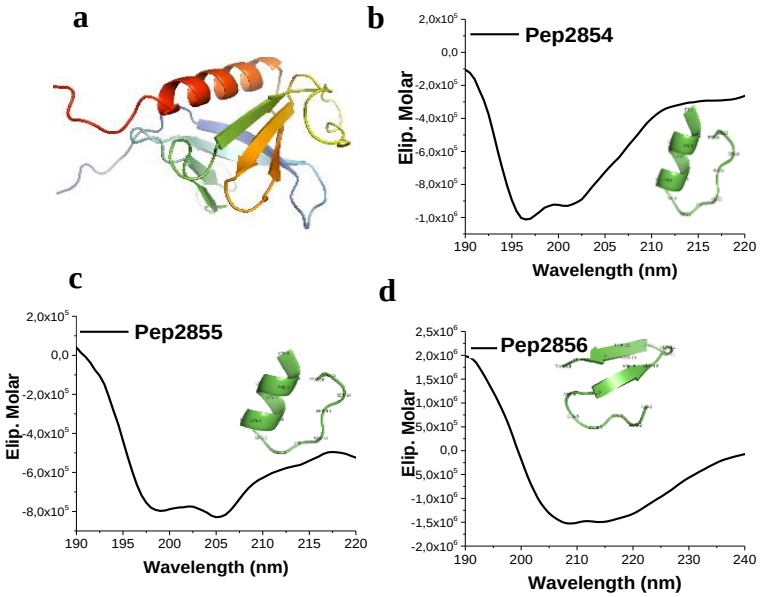


Fig. 1. Circular dichroism spectra of the tendency of secondary structures of each synthetic peptide in 30% v/v in trifluoroethanol with a peptide concentration of 0.1 mg mL^{-1}. **a**) 2D structure of a lectine reported with PDB code: 2DHI. **b**) Pep2854 2D-folding with an α-helix structure tendency. **c**) Pep2855 2D-folding with an α-helix structure stronger tendency. **d**) Pep2856 2D-folding with a beta-sheet tendency.

This finding gives excellent reliability and robustness to the molecular docking between these peptides and the glycoproteins under study. Bo et al. shows that the peptide sequences of proteins often have intrinsic propensities for the formation of their native conformations [31] which is in consistent with our designed peptide that keeps the motifs structural from Lectins.

The most relevant physicochemical properties (i.e., molecular weight, net charge, isoelectric point, etc.) of the peptidomimetics are presented in **Table 1**. As for the molecular dynamics performed, the CABS-Dock server-generated ten top-ranked model structures of both the sAP-peptide and the PPG-peptide complex. The docking poses predict the binding orientations of a ligand to the receptor active site, many times defined by the user to evaluate stabilizing interactions such as hydrogen bonds, salt-bridges, and hydrophobic contacts possible in the pairs of molecules using PDB files [32]. The computational experiment allowed the full flexibility of the novel synthesized peptides and a small fluctuation of the receptor backbone to explore the binding position according to the score function energies by calculating the root mean square deviation (RMSD) of each pose [33].

Table 1. Physicochemical properties of synthetic peptides designed from lectins mannose-galactose binding proteins.

ID Peptide	Sequence	MW (Da)	C*	pI	2D-folding	#AA
Pep2854	HYSRTENMGTSNAC	1569.68	+1	8.58	Alpha-helix	14
Pep2855	KTFLKICKRPYVPSEP	1905.31	+4	10.5	Alpha-helix	16
Pep2856	INKQGDVTVLSNGNTQLT	1901.08	+1	10.14	Betha-sheet	18

*Net charge at pH 7.0; isoelectric point (pI); Number of amino acidic residues (AA).

Three different RMSD classifications for docking solutions were used as a reference: i) right solution when RMSD ≤ 2.0 Å; ii) acceptable solution when RMSD is between 2.0 and 4.0 Å; and iii) wrong solution when RMSD ≥ 4.0 Å [34]. The present work was oriented by prior knowledge [35,36] of the interactions that could take place between three peptides homologous to lectin and two different glycoconjugates as shown in **Fig. 2**, the partial loss of the 2D conformation of the peptides can be seen when they are approaching the sAP region. The more frequent contacts were between sAP residues Arg11, Ser 12 and Asn17 and Pep2854 residues Asn7, Ser11, Asn11 at a high-frequency level (0.06-0.1) according to the contact maps.

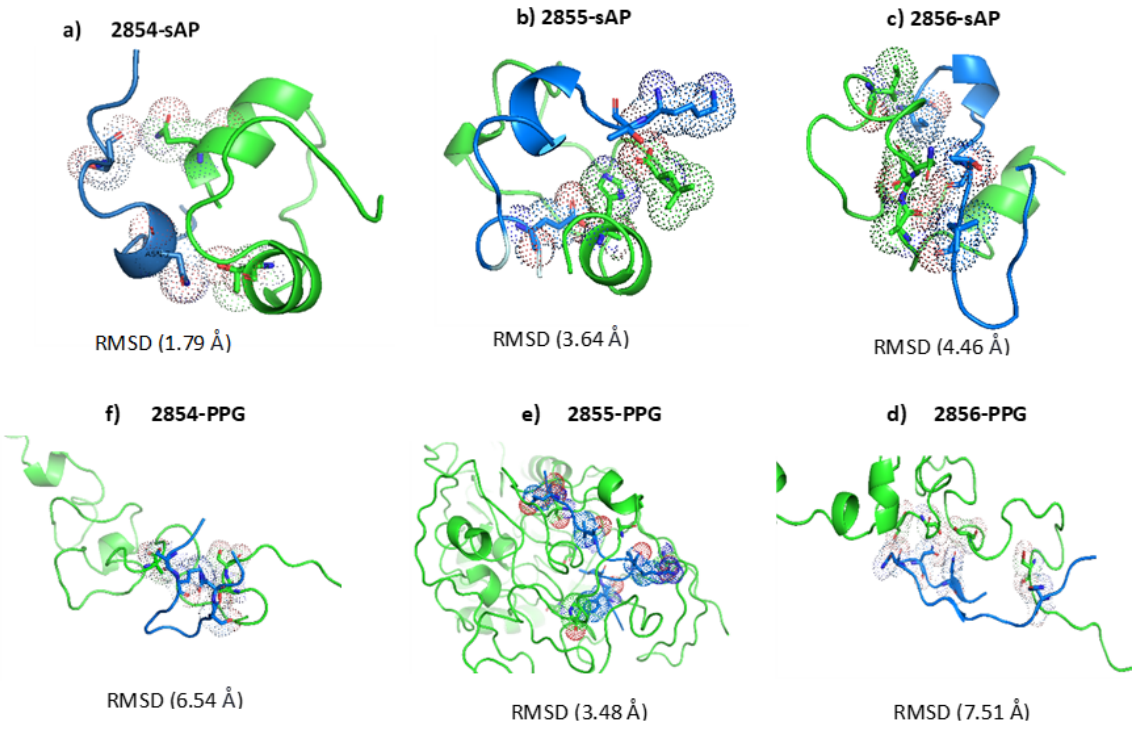


Fig. 2. Top-ranked models obtained from CABS-Dock docking with the peptide-RMSD values. The structures represent each of the peptides bound close to a sAP fragment of the conservative region corresponding to MVQVVHRHGARSALNDNTTEICGTLYPC **(a-c)**. The structures represent each of the peptides bound close to the PPG binding at the conservative region (Uniprot code: A4HM87$_{1030\text{-}1450}$) (**d-f**). The target region of glycoprotein is coloured in green and the peptide is coloured in blue. The dotted region represents the most likely site for binding interaction according to the contact map.

For the dynamic molecular trajectories, the smallest RMSD of atomic coordinates value was 1.79 Å in the docking for trajectories of the Pep2854-sAP pair, thus validating the structure-based design process. We focused on the clustering based on the fewer RMSD values released to the peptide bond in the proximity of the binding site on the bioreceptor. For PPG in **Fig. 2**, it was hypothesized that Ser275, Ser289, and Ser363 of the protephosphoglycans domain are the key binding sites to the cationic peptides through Lys, Arg and Glu residues, for this case most interactions were salt bridges. For Pep2855-PPG molecular dynamics, the smallest calculated RMSD value was 3.48 Å; this value is on the borderline of a bad result. However, this must be considered since the calculation of RMSD increased significantly with the molecular size of the MD structures; then, the RMSD became on data with many attributes when the high dimensionality causes that the algorithms based on the clustering methods to calculate the trajectory poses to fail [37]. Hence, these data are considered as good approximations, which were complemented and correlated with the affinity counters obtained by subsequent SPR experiments.

Determination of binding affinities by Surface Plasmon Resonance biosensor analysis

SPR biosensor technique plays a prominent role in biomolecular interactions characterization due to its ability to detect and monitor molecular binding events in real time without the use of labels. A home-made SPR biosensor working in *Kretschmann* configuration was employed for the analysis and determination of the binding affinities and kinetic parameters of the interactions between the target proteins and the new peptides. The working principle of the SPR biosensor is based on the high sensitivity of an evanescent plasmonic field generated at the interface of a nanometric metal layer (i.e. 50 nm of gold) and the dielectric medium when the light strikes at a specific angle. The properties of the evanescent field depend significantly on the refractive index of the medium whereby the small changes due to molecular interactions can be measured, as well as the formation of molecular pairs (target-bioreceptor) that takes place on the sensor surface. The SPR analysis requires that one of the studied molecules is immobilized on the gold surface chip. The strategy used was the formation of the covalent amine coupling between the N-Hydroxy succinimide (NHS-activated) of a COOH-functional alkanethiol self-assembled monolayer (SAM) and the primary amine group present in each peptide chain [38] (see **Fig. 3**). To facilitate the homogeneous distribution of the peptide monolayer on the surface and minimize steric hindrance issues, hydroxyl-functional alkanethiols were used as a lateral spacer in the SAM.

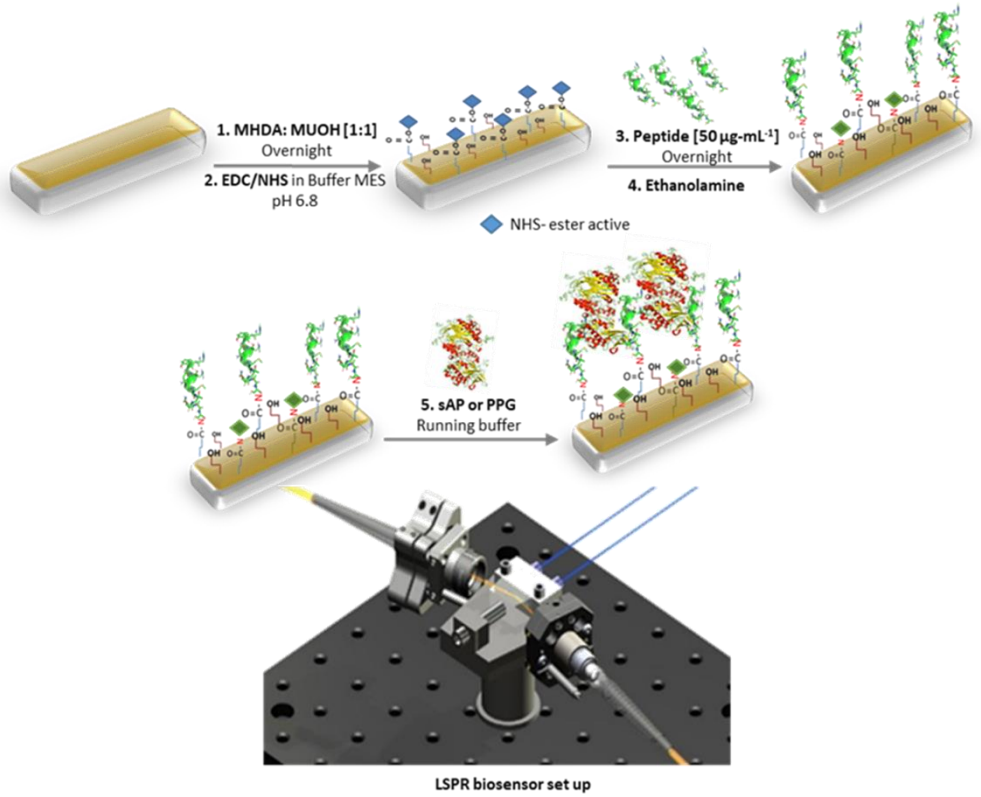


Fig. 3. Biofunctionalization strategy onto a plasmonic sensor chip for SPR analysis of the binding interaction between each of the synthetic peptides with both sAP and PPG.

We independently evaluated the interaction of sAP and PPG with the three peptidomimetics, previously immobilized on the sensor chip. Results shown in **Fig. 4** correspond to the fitting of the experimental sensorgram to the theoretical kinetic parameters and the statistical parameter for all fitted curves (Chi and R-square).12

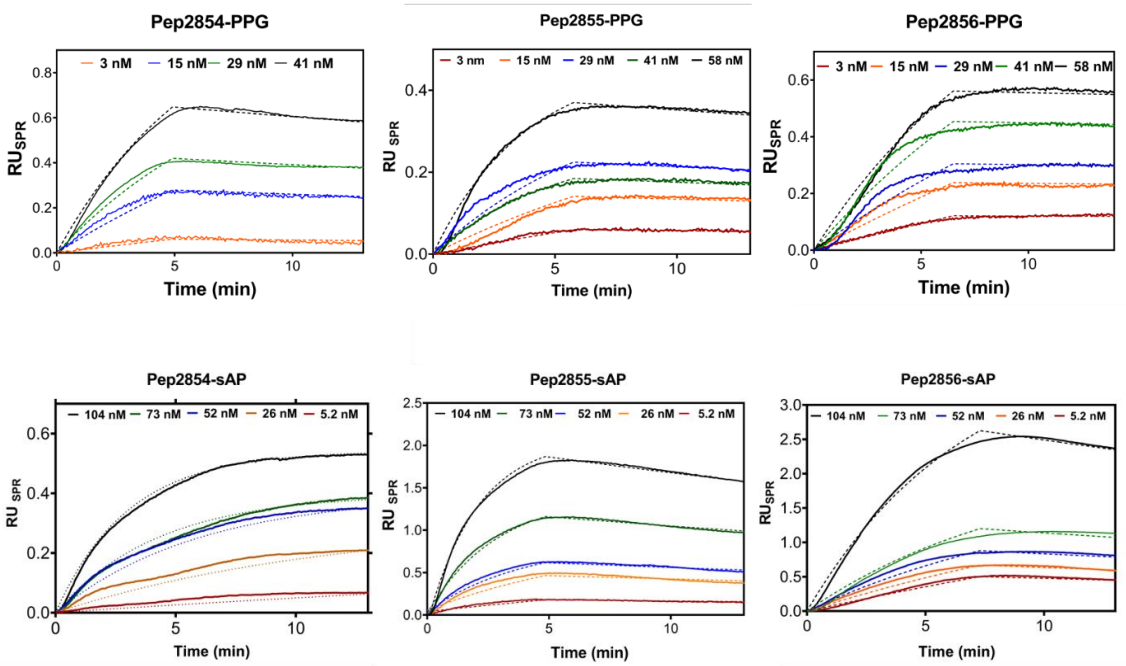


Fig. 4. SPR analysis of peptidomimetics interactions with both sAP and PPG. The sAP was tested in the concentration range from 5.2 to 104 nM, while for PPG, the concentration range was from 3.0 to 58 nM, enough to cover the kinetic curves. The sensorgrams are shown in continuous lines, while fitting curves are shown in dotted line.

The sensorgrams resulting from the injection of the sAP and PPG solution over the SAM without immobilized peptide showed the full recovery of the baseline after the injection of the targets, confirming the absence of nonspecific adsorptions to the sensor surface at the better conditions of peptide immobilization (Supporting Information **Figure. S2**). In the **Table 2-3** from the fitting equations, it is possible to extract both kinetics (k_{on} and k_{off}) and affinity parameters for the interaction. In terms of kinetics, the association constant (k_{on}) indicates the rapidness of the biomolecular interaction but, importantly, the dissociation constant (k_{off}) is a measure of the stability of the complex. In this sense, the Pep2854-sAP and Pep2856-PPG complexes are the most stable ones and their k_{off} dissociation constant showed small values of 0.004 and 0.001 s^{-1}, respectively. This observation can be interpreted as the fraction of the complex that dissociates every second, which is related to a longer half-life of these two molecular complexes. Regarding the k_{on} measurement, we observed that Pep2855 quickly bounds to the two target molecules, showing similar k_{on} values for both sAP ($4.49x10^{-6}$ M s^{-1}) and PPG ($4.4x10^{-6}$ M s^{-1}), however these interactions were not the most stable. We infer from this finding that both Pep2854 and Pep2856 not only have a high affinity for the studied glycoproteins but also exhibit a distinct specificity for one of the targets. Affinity is the binding strength of a molecule to its ligand, though the binding

is a reversible process, the measurement of the reaction rate constants can be used to define an equilibrium or affinity constant ($1/K_D$), so the smaller the K_D value, the higher the affinity of the peptide for its target [38].

Table 2. Kinetic parameters of the interaction of synthetic peptides with sAP

ID Peptide	K_D (nM)	k_{on} ($x10^6$ M-s^{-1})	k_{off} (s^{-1})	R-square ($R^2 \approx 1$)	Chi (Chi ≈ 0)
Pep2854	1.47	3.15	0.004	0.995	0.012
Pep2855	4.42	4.49	0.019	0.997	0.025
Pep2856	15.6	1.26	0.020	0.995	0.044

Table 3. Kinetic parameters of the interaction of synthetic peptides with PPG

ID Peptide	K_D (nM)	k_{on} ($x10^6$M-s^{-1})	k_{off} (s^{-1})	R-square ($R^2 \approx 1$)	Chi (Chi ≈ 0)
Pep2854	5.0	2.6	0.014	0.995	0.014
Pep2855	2.7	4.4	0.012	0.993	0.008
Pep2856	1.76	1.8	0.001	0.987	0.019

All peptides showed K_D values in the nanomolar range, evidencing the high affinity for sAP and PPG. The molecular pairs, Pep2854-sAP and Pep2856-PPG, registered the smallest K_D values equal to 1.47 nM and 1.76 nM, respectively, as shown in Table 2-3. One of the most significant observation is that the interaction of the Pep2856-PPG was 9-fold stronger than Pep2856-sAP, presumably because the positive charge of Pep2856 (+4) increases the electrostatic interaction with the negative groups in the PPG molecule at sites different to the active site. These affinity values are like the K_D values of high-affinity antibodies, generally considered to be in the range of 1 to 10^{-3} nM [39]. Nevertheless, synthetic peptides show some better properties than antibodies, such as their simple and robust production at large scales and their high biochemical stability, therefore offering an interesting alternative for their use in diagnostic tests and devices. Finally, we demonstrate the specificity of the peptides towards the molecules tested. The interaction by the peptides is not significant since an increase in the signal is observed due to changes in the refractive index of the medium due to the bulky effect of BSA when it passes through the sensor surface (see **Figure S5**).

Conclusions

We have designed and synthesized three high-affinity peptidomimetics based on the galactolectins family, which can recognize Leishmania sAP and PPG proteins, considered as potential targets for the diagnosis of the infection. The dynamic molecular strategy employed allowed to model the most likely interactions between peptides and the targets selected, finding RMSD values within the acceptable limits for theoretical calculations

(~3.0 Å). Pep2854 and Pep2856 exhibited the highest affinity and specificity for sAP (K_D of 1.47 nM) and PPG (K_D of 1.76 nM). These results support the hypothesis that peptides based on a rational design with the ability to occupy the binding site of a target glycoprotein can be used as high affinity bioreceptors, as an alternative to large biomolecules as antibodies with limitations in terms of production, biochemical stability and robustness. These peptides can be employed in the development of new diagnostic tools for cutaneous leishmaniasis, especially for the construction of point-of-care biosensor devices that enable rapid and accurate detection of the infection in decentralized and low-resource settings.

Conflicts of interest

There are no conflicts to declare.

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