Leishmania (Viannia) braziliensis: insights on subcellular distribution and biochemical properties of heparin-binding proteins

LUZIA MONTEIRO DE CASTRO CÔRTES[1], MIRIAN CLAUDIA DE SOUZA PEREIRA[2], FRANCISCO ODÊNCIO RODRIGUES DE OLIVEIRA JUNIOR[1,2], SUZANA CORTE-REAL[3], FRANKLIN SOUZA DA SILVA[1], BERNARDO ACÁCIO SANTINI PEREIRA[1], MARIA DE FÁTIMA MADEIRA[6], MARCIA TEREZINHA BARONI DE MORAES[5], REGINALDO PEÇANHA BRAZIL[4] *and* CARLOS ROBERTO ALVES[1]*

[1] *Laboratório de Biologia Molecular e Doenças Endêmicas*, [2] *Laboratório de Ultraestrutura Celular*, [3] *Laboratório de Biologia Estrutural*, [4] *Laboratório de Bioquímica e Fisiologia de Insetos and* [5] *Laboratório de Virologia Molecular, IOC – FIOCRUZ, Av. Brasil 4365, Rio de Janeiro, CEP 21040-360, Brasil*
[6] *Laboratório de Vigilância em Leishmanioses, IPEC – FIOCRUZ, Av. Brasil 4365, Rio de Janeiro, CEP 21040-360, Brasil*

(*Received 29 July 2011; revised 15 September and 23 September 2011; accepted 27 September 2011; first published online 7 November 2011*)

SUMMARY

Leishmaniasis is a vector-borne disease and an important public health issue. Glycosaminoglycan ligands in *Leishmania* parasites are potential targets for new strategies to control this disease. We report the subcellular distribution of heparin-binding proteins (HBPs) in *Leishmania (Viannia) braziliensis* and specific biochemical characteristics of *L. (V.) braziliensis* HBPs. Promastigotes were fractionated, and flagella and membrane samples were applied to HiTrap Heparin affinity chromatography columns. Heparin-bound fractions from flagella and membrane samples were designated HBP F_f and HBP M_f, respectively. Fraction HBP F_f presented a higher concentration of HBPs relative to HBP M_f, and SDS-PAGE analyses showed 2 major protein bands in both fractions (65 and 55 kDa). The 65 kDa band showed gelatinolytic activity and was sensitive to inhibition by 1,10-phenanthroline. The localization of HBPs on the promastigote surfaces was confirmed using surface plasmon resonance (SPR) biosensor analysis by binding the parasites to a heparin-coated sensor chip; that was inhibited in a dose-dependent manner by pre-incubating the parasites with variable concentrations of heparin, thus indicating distinct heparin-binding capacities for the two fractions. In conclusion, protein fractions isolated from either the flagella or membranes of *L. (V.) braziliensis* promastigotes have characteristics of metallo-proteinases and are able to bind to glycosaminoglycans.

Key words: *Leishmania (Viannia) braziliensis*, glycosaminoglycans, heparin, heparin-binding protein, surface plasmon resonance, metallo-proteinase.

INTRODUCTION

The genus *Leishmania* (Kinetoplastida: Trypanosomatidae) includes species that are causative agents of leishmaniases, which are widely spread anthropozoonotic diseases in tropical and subtropical regions. In Brazil, *Leishmania (Viannia) braziliensis* is the main species involved in the cutaneous and mucocutaneous forms of the disease (Reithinger *et al*. 2007). In the southeast and northeast regions of Brazil, this species is mainly transmitted by the sand flies *Lutzomyia (Nyssomyia) intermedia* and *Lutzomyia (Nyssomyia) whitmani* (Rangel and Lainson, 2003).

During its life cycle, *Leishmania* parasites alternate between promastigote and amastigote forms within the invertebrate and vertebrate hosts, respectively. The former forms survive in the sand fly digestive tract lumen, whereas the latter are found in the parasitophorous vacuoles of macrophages (Bates and Rogers, 2004). In the case of *L. (V.) braziliensis*, a peripylarian parasite, its development begins in the hindgut of the insect followed by migration to the anterior midgut and mouth parts (Lainson and Shaw, 1987).

The cell surfaces of *Leishmania* spp. present molecules that are related to specific activities during the life cycle of the parasite and contribute to survival in harsh surroundings (Descoteaux and Turco, 1999). These surface molecules not only afford protection to the parasite within the insect vectors and vertebrate hosts but also provide specificity for their interactions with cells in the sand fly gut and with mononuclear phagocytic cells of mammals. The most intensely studied molecules located on the surfaces of promastigotes and amastigotes are the glycoconjugate lipophosphoglycan (LPG) and the metallo-proteinases (Moody, 1993). *Leishmania* spp.

* Corresponding author: LABIMDOE, IOC, Fiocruz. Av. Brasil 4365, CP 926, 21040-360, Manguinhos, Rio de Janeiro, RJ, Brazil. E-mail: calves@ioc.fiocruz.br

Parasitology (2012), **139**, 200–207.
doi:10.1017/S0031182011001910

also produce a number of less-abundant surface molecules: glycosylinositol phospholipids (McConville *et al*. 1990), a promastigote surface antigen-2 complex of glycoproteins (Murray *et al*. 1989; Symons *et al*. 1994), a glycoprotein of approximately 46 kDa (Kahl and McMahon-Pratt, 1987) and 2 cysteine-proteinases of 63 and 43 kDa (Rebello *et al*. 2009). These molecules share the common trait of attaching to the plasma membrane via glycosylphosphatidylinositol lipid anchors.

The activity of these metallo-proteinase surface components has been associated with the hydrolysis and inactivation of immunoglobulin G, the inactivation of factor C3b of the complement C3bi in the mammalian host (Brittingham *et al*. 1995), and the protection of promastigotes from trypsin and chymotrypsin in the gut of the insect vector (Pimenta *et al*. 1997). Although LPGs have commonly been identified as having a life cycle-maintaining role, they can also act on the vertebrate host (i.e., influencing the innate and acquired immune responses and subverting the functions of the macrophage – Becker *et al*. 2003; Brandonisio *et al*. 2004; Lodge and Descoteaux, 2008; Soong, 2008) and on the insect vector (i.e., protecting the parasite from the proteolytic activities of the blood-digesting midgut, participating in the attachment to the gut wall, inducing the production of chitinases that degrade the stomodeal valve of the sand fly and influencing the secretion of aneuropeptide that arrests midgut and hindgut peristalsis – Sacks and Kamhawi, 2001; Kamhawi, 2006).

Metacyclic forms of *L. (V.) braziliensis* LPGs have also been reported to display differential attachment to the midgut of *Lutomyia (Nyssomyia) whitmani* and *Lutzomyia (Nyssomyia) intermedia* sand flies (Soares *et al*. 2010). This observation has been related to an upregulation of β-glucose residues in the LPG repeated units during metacyclogenesis of this parasite (Soares *et al*. 2005).

There is evidence that glycosaminoglycans, such as heparin, influence the development of *L. (L.) major* in the gut of the vector, increasing the parasitic load in experimentally infected insects (Volf *et al*. 2001). It has also been reported that specific receptors on the surface of *Leishmania* spp. are able to bind glycosaminoglycans with structures similar to heparin present in host tissues. Such receptors are known as heparin-binding proteins (HBPs), and these molecules can influence the parasite-host interactions during infection.

Experiments performed using promastigotes of *Leishmania (Leishmania) donovani*, for example, have shown that HBPs can be found on the surface of the parasite and are related to the inhibition of protein kinase C (Mukhopadhyay *et al*. 1989). HBPs have been associated with the infective forms of *L. (L.) donovani*, as they predominate on the surface of stationary-phase promastigotes from recently isolated samples. However, subsequent passages of parasites in culture have led to the loss of the heparin-binding abilities of the parasites (Butcher *et al*. 1992; Kock *et al*. 1997).

HBPs have also been described in other *Leishmania* species, specifically *Leishmania* (*Leishmania*) *amazonensis* and *Leishmania* (*Leishmania*) *major*; however, contrary to what has been observed for *L. (L.) donovani*, in these species those proteins appear to be predominant in amastigotes (Love *et al*. 1993).

In addition, HBPs can influence the parasitic attachment to the gut of the insect vector because hydrophobic HBPs from *L. (V.) braziliensis* have been shown to possess the physicochemical potential to bind to *L. (N.) whitmani* and *L. (N.) intermedia* gut cells (Azevedo-Pereira *et al*. 2007).

Due to the lack of information on HBPs in *L. (V.) braziliensis*, we present new data regarding the subcellular distribution, proteolytic activity and binding kinetics of HBPs from this parasite.

MATERIALS AND METHODS

Chemicals

Detergents {Tween 20, Triton X-100 (TX-100), and 3-[(3-cholamidopropyl)-dimethylammonium]-1-propanesulfonate (CHAPS)}, heparin sodium salt, biotinylated heparin, gelatin, bovine serum albumin (BSA), penicillin, proteinase inhibitors [trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), phenylmethanesulfonyl fluoride (PMSF), 1,10-phenanthroline (o-phe), pepstatin A (Pep A), and p-phenylenediamine], reducing agents [dithiothreitol (DTT) and β-mercaptoethanol (β-ME)] were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). A heparin-Sepharose column (HiTrap-Heparin; 1·5 × 2·5 cm) was purchased from GE Healthcare. Fetal calf serum (FCS) was purchased from Cultilab S/A (Brazil). Brain heart infusion (BHI) medium was purchased from Difco (Detroit, USA). Epon resin was purchased from Hexion Specialty Chemicals, Inc. (US). The electrophoresis reagents were purchased from Bio-Rad Laboratories Inc. (US). Pre-Stained™ Plus Protein Ladder was purchased from Fermentas Life Sciences (US). Amicon Centriprep YM-10 filter devices were purchased from Millipore (Billerica Inc, MA, USA). Chemiluminescence luminol reagent-ECL kit was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other reagents were of analytical grade or better.

Parasite strain and culture

Infective promastigotes of *L. (V.) braziliensis* (strain MCAN/BR/1998/619) were maintained at 28 °C as a stock culture in Novy, MacNeal and Nicolle medium and subcultured every 4 days. Promastigote cultures

were grown in Brain Heart infusion medium supplemented with 10% heat-inactivated FCS until a density of 1×10^8 cells/ml was obtained.

Subcellular fractionation

Subcellular fractions enriched for flagella or surface membranes were obtained by centrifugal fractionation as previously described (Morgado-Diaz *et al*. 2005). Briefly, after 4 days of culturing, promastigotes were washed twice (3800 ***g***, 10 min, 4 °C) in phosphate-buffered saline, pH 7·2, (PBS). The remaining pellet was then washed twice with 10 mM Tris-HCl (pH 7·2) buffer containing 1 M NaCl, 0·2 M K_2HPO_4 and 0·5 M $MgCl_2$. The pellet was then resuspended in 10 mM Tris-HCl, pH 7·5, containing 0·05 M sucrose (**S** buffer; 10 ml/g of cells) and disrupted in 1% CHAPS with 40–80 strokes of a Dounce-type homogenizer. Isotonic conditions were restored by adding sucrose to reach a final concentration of 0·25 M. Cell lysates were centrifuged (10 min, 4300 ***g***, 4 °C), and the supernatant was collected and centrifuged (15 min, 12 000 ***g***, 4 °C). The pellet from the final centrifugation constituted the flagellar fraction (F_f), whereas the supernatant was centrifuged again (45 min, 35 000 ***g***, 4 °C) to obtain the pellet that comprised the membrane fraction (M_f).

Electron microscopy

The ultrastructural composition of subcellular fractions was determined as previously described (Morgado-Diaz *et al*. 2005). Briefly, subcellular fractions were fixed with 2·5% glutaraldehyde in a 0·1 M sodium cacodylate buffer, pH 7·2, for 1 h at 4 °C and post-fixed with a 1% osmium tetroxide (OsO_4) solution for 1 h at 4 °C. Samples were dehydrated in a graded series of acetone and embedded in PolyBed 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed using a JEOL-1011 (JEOL UK – Welwyn Garden City, Hertfordshire, UK) transmission electron microscope.

Affinity chromatography

The soluble proteins from F_f and M_f were dialysed against an equilibrium buffer (10 mM sodium phosphate, pH 7·0, and then passed through a HiTrap-Heparin column previously equilibrated in the same buffer. The column was washed with the equilibrium buffer adjusted with 0·5 M NaCl, and the bound proteins were eluted using the equilibrium buffer adjusted with 2·0 M NaCl at a flow rate of 2 ml/min. The eluted fractions were concentrated using a Centriprep filter device, and the protein concentration was determined as previously described (Lowry *et al*. 1951).

Electrophoresis and Western blot analysis

Soluble proteins (~10 μg) were resolved using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) and silver staining, as previously described. Electrophoresis was performed at 25 °C in a Mini Protean II system (Bio-Rad Laboratories, USA). For Western blot assays, proteins were resolved using SDS-PAGE and then transferred onto 0·22 μm nitrocellulose membranes, as previously described (Azevedo-Pereira *et al*. 2007). Non-specific binding sites were blocked (4 °C for 16 h) using a solution of 5% skimmed milk (w/v) in PBS containing 0·5% Tween 20. The blots were washed 3 times with PBS containing 0·05% Tween 20 (PBST) and incubated (37 °C, 1 h) with biotinylated heparin diluted 1:500 in PBST. After 6 washes with PBST, the blots were incubated (37 °C, 1 h) with 1:1000 streptavidin conjugated to horseradish peroxidase in PBST. After 6 washes with PBST, the complex was revealed by chemiluminescence.

Zymographic assays

Protease activities of the HBPs (in samples of 5 μg) were determined using a gelatin substrate (0·1%) in 12% polyacrylamide gels (Heussen and Dowdle, 1980; Alves *et al*. 1993). Electrophoresis under denaturing conditions (using SDS) was performed at 4 °C in a Mini Protean II system and, subsequently, the gels were washed with 2·5% Triton X-100 under agitation (60 min, 25 °C) and incubated (16 h, 37 °C) in the appropriate buffer for each proteinase class (aspartic-proteinase: 10 mM sodium acetate buffer, pH 3·0; cysteine-proteinase: 10 mM sodium acetate buffer, pH 5·5, containing 1 mM DTT; serine-proteinase: 10 mM Tris-HCl, pH 7·5; metallo-proteinase: 10 mM Tris-HCl, pH 8·0). Incubations were performed in the presence or absence of inhibitors for each proteinase class (aspartic-proteinase: 1·0 μM Pep A; cysteine-proteinase: 10·0 μM E-64; serine-proteinase: 1·0 mM PMSF; and metallo-proteinase: 1·0 mM o-phe). The hydrolysis of gelatin was detected by staining the gels with 0·1% (w/v) Coomassie blue prepared in a methanol:acetic acid: water (3:1:6, v/v/v) solution.

Surface plasmon resonance (SPR) assays

SPR assays were performed using sensor chips that presented a carboxyl surface (COOH) coated with immobilized neutravidin (Biocap; Nomadics, USA). The sensor chip surface was further covered with biotinylated heparin (0·5 μg) and used to detect the presence of HBPs in each fraction (2 μg), BSA (1·0–0·01 μg) or in whole promatigotes ($1{\cdot}4 \times 10^5$ cells). Alternatively, promastigotes were pre-incubated (2 h, 4 °C) with different concentrations of heparin

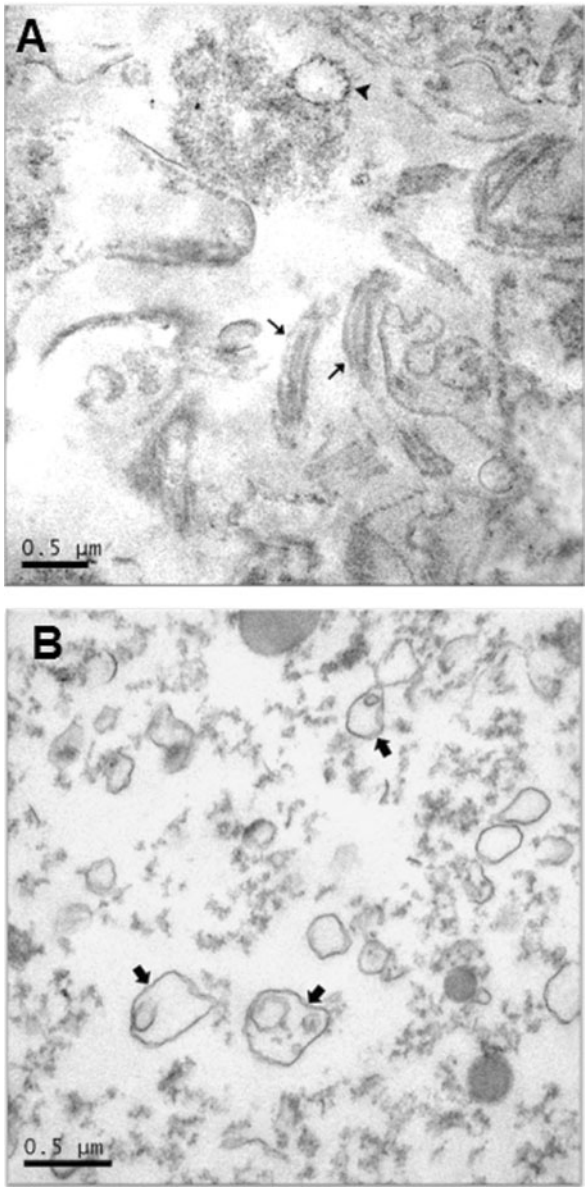

Fig. 1. Ultrastructural analysis of flagellar – F_f – (A) and membrane – M_f – (B) subcellular fractions of *Leishmania (V.) braziliensis* promastigotes obtained after differential centrifugation. The F_f was recognized by flagellum fragments (←) and flagellar membrane (▶), and the M_f by spherical membrane-bound vesicles (⬅). These images are representative of 3 independent experiments.

(1·0 μg/ml, 0·1 μg/ml and 0·01 μg/ml) in PBS to assess specific binding inhibition. Prior to interaction with the sensor chip surfaces the promastigotes were fixed (1 h, 4 °C) with 1% paraformaldehyde and washed 3 times by centrifugation (800 ***g***, 10 min, 4 °C) in PBS. SPR assays were performed at 25 °C with 100 μl of material injected under a flow rate of 10 μl/min. The binding assays were performed in PBS and registered in real time using a sensorgram, where changes in the SPR angle (θspr) were measured as resonance units (RU). Such changes were proportional to the concentration of bound proteins or the number of cells attached to the sensor chip surfaces. Resonance signals of the samples were analysed after subtraction of the RU values from the reference channel. Association (Ka) and dissociation (Kd) constants were measured and used to calculate the affinity constant of the fraction samples (KD). SPR experiments were conducted in an optical biosensor SensiQ Pioneer (Icx Nomadics, USA), and the data were analysed using Qdat software (Icx Nomadics, USA).

RESULTS

Ultrastructural characterization of the subcellular fractions of promastigotes

Subcellular fractions were obtained from promastigotes in the early stationary growth phase. Differential centrifugation was used for the enrichment of flagella (F_f) and membrane (M_f) fractions (Fig. 1A and B, respectively). F_f was assessed by the presence of typical flagellum fragments and flagellar membranes, whereas M_f was assessed by the predominance of spherical membrane-bound vesicles, using transmission electron microscopy.

Heparin-binding proteins from F_f and M_f fractions of promastigotes

We assessed the heparin-binding properties in proteins from both aforementioned isolated subcellular fractions. These assays yielded an estimative value of 0·04 ± 0·001 mg of HBPs in F_f, corresponding to 13·3% of the total fraction of proteins (0·3 ± 0·1 mg), whereas HBPs in M_f yielded 0·05 ± 0·001 mg of HBPs, corresponding to 8·3% of the total M_f proteins (0·6 ± 0·02 mg).

The protein profiles of F_f and M_f (and their correspondent heparin-binding fractions) were analysed by SDS-PAGE. Western blot analysis with F_f and M_f indicated that these fractions present complex and distinct band profiles ranging from 65·0 to 17·0 kDa. Surprisingly, denaturant electrophoresis assays with samples from both subcellular fractions after elution from the heparin-affinity columns showed 2 main protein bands with relative molecular masses of approximately 65·0 and 55 kDa, as revealed by silver staining (Fig. 2).

Proteinase properties of HBPs from M_f and F_f fractions

Our results indicate that the 65 kDa-protein band, from both HBP fractions, has a major gelatinolytic activity within a pH range between 5·5 and 8·0. A band with a minor gelatinolytic activity was also observed around 49 kDa in HBP M_f. The activity of these bands was sensitive to inhibition by 1,10-phenanthroline, and no inhibition was evident in gels incubated with other proteinase inhibitors, thus suggesting metallo-proteinase-like activity (Fig. 3).

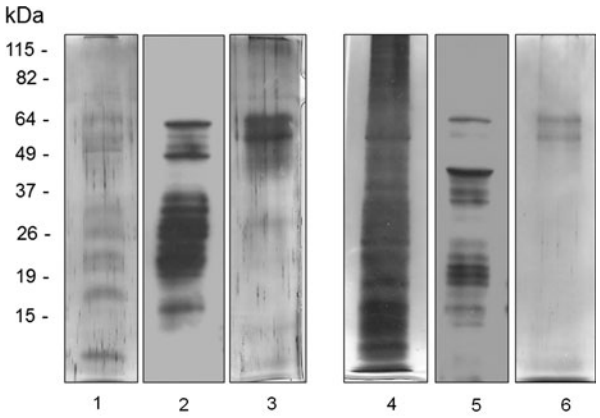

Fig. 2. Electrophoresis assays of samples from *Leishmania (V.) braziliensis*. Samples from flagellar (F_f) and membrane (M_f) fractions collected prior to (1 and 4, respectively) or after heparin-Sepharose column purification (HBP F_f (3) and HBP M_f (6)) were applied into each slot, submitted to SDS-PAGE and revealed by silver staining. In addition, Western blotting using biotinylated heparin as marker and revelation by chemiluminescence was performed with F_f (2) and M_f (5) to detect HBPs. Molecular mass markers (in kDa) are indicated on the left. These results are representative of 5 independent experiments.

Heparin-binding assays with Leishmania (V.) braziliensis *proteins*

SPR assays were designed to assess the presence of heparin ligands on *L. (V.) braziliensis* promastigote surfaces and to directly quantify these ligands in parasite subcellular fractions obtained by affinity chromatography. Kinetic constants obtained during the binding of biotinylated heparin onto a sensor chip surface characterized the specificity of this immobilization ($Ka = 2{\cdot}4 \times 10^5\ M^{-1}\ s^{-1}$; $Kd = 0{\cdot}079\ s^{-1}$; $KD = 320$ nM).

The presence of HBPs on the surface of promastigotes was confirmed by the sensorgram display of association and dissociation values of $33{\cdot}0 \pm 2{\cdot}0$ RU and $14 \pm 1{\cdot}0$ RU, respectively, following the injection of parasites onto the sensor chip surface. These values of association and dissociation are 1·6-fold higher than those observed after the injection of heparin (baseline), thus indicating binding of parasites to the immobilized heparin on the sensor chip surface (Fig. 4).

The specificity of parasite binding to heparin was confirmed by additional assays in which the promastigotes were incubated with increasing concentrations of heparin prior to being injected on the sensor chip surface. In these assays, it was possible to detect a dose-dependent inhibition of promastigotes binding to immobilized heparin with decreasing RU values of 48%, 39%, and 33% inhibition when parasites were incubated with 1·0, 0·1 and 0·01 μg/ml of heparin, respectively, as shown in Fig. 4.

We also analysed the capacity of proteins from HBP F_f and HBP M_f to bind to heparin at neutral pH. The analysis of the dissociation RU values indicated that HBP F_f contains more HBPs (445 ± 30 RU, equivalent to $0{\cdot}4$ ng/mm^2 of proteins on the sensor chip surface) than HBP M_f (175 ± 20 RU, equivalent to $0{\cdot}1$ ng/mm^2). The RU values observed for the HBP F_f and M_f fractions were 53-fold and 20-fold higher, respectively, than the RU value observed after the immobilization of heparin (baseline). The binding of HBP F_f samples to immobilized heparin was also observed to be stronger than that of HBP M_f with lower dissociation rates (Fig. 5). As expected, the negative controls using BSA showed very low dissociation RU values at 3 different concentrations ($1{\cdot}0\ \mu g = 2{\cdot}0 \pm 0{\cdot}3$ RU, $0{\cdot}1\ \mu g = 1{\cdot}2 \pm 0{\cdot}1$ RU and $0{\cdot}01\ \mu g = 0{\cdot}4 \pm 0{\cdot}3$ RU).

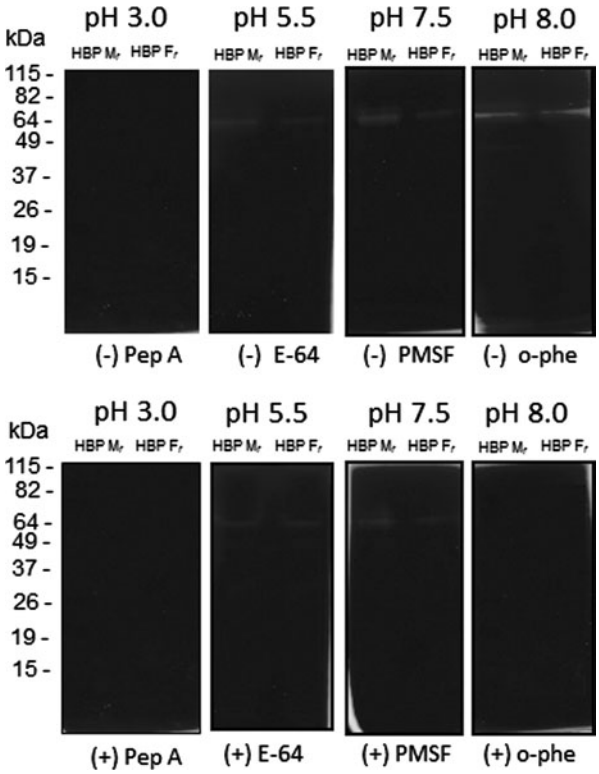

Fig. 3. Measurement of proteinase activity in *Leishmania (V.) braziliensis* HBPs by gelatin-SDS-PAGE. After electrophoresis of samples eluted from a heparin-Sepharose column (membrane proteins – HBP M_f; flagellum proteins – HBP F_f), gels were incubated with different buffers (pH 3·0, pH 5·5, pH 7·5 and pH 8·0) in the absence (−) or presence (+) of specific inhibitors for different classes of proteinases: Pep A, E-64, PMSF and o-phe. The gelatinolytic bands were detected by negative staining with a Coomassie blue solution. Molecular mass markers are indicated (kDa). These results are representative of 4 independent experiments.

DISCUSSION

The surfaces of protozoan parasites, such as those belonging to the genus *Leishmania*, regulate interactions with the extracellular environment and are responsible for the uptake of nutrients and signalling

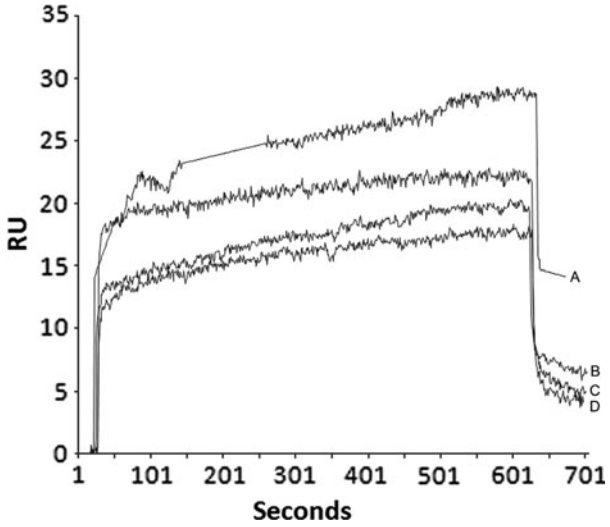

Fig. 4. Analysis of HBPs on the surfaces of *Leishmania (V.) braziliensis* promastigotes by surface plasmon resonance biosensing. Sensor chips were covered with biotinylated heparin, and promastigotes were passed onto the chip surface. The parasites were assayed without pre-incubation with heparin (A) or with pre-incubation with 0·01 μg (B), 0·1 μg (C) or 1·0 μg (D) of heparin. The data are presented as resonance units (RU) and are representative of 2 independent experiments.

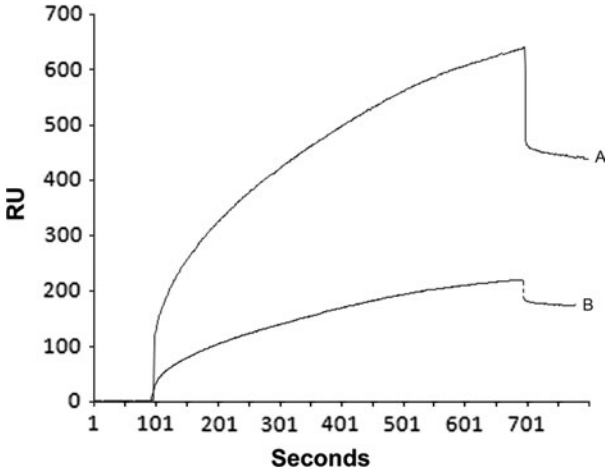

Fig. 5. Analysis of the interactions between heparin and HBP-enriched fractions from *Leishmania (V.) braziliensis* promastigotes by surface plasmon resonance biosensing. The interaction kinetics of HBP F_f (A) and HBP M_f (B) with biotinylated heparin immobilized on a sensor chip were assessed. The data are presented as resonance units (RU) and are representative of 2 independent experiments.

pathways. In this context, we have previously proposed the potential of HBPs from *L. (V) braziliensis* promastigotes to mediate parasitic adhesion to proteins from the guts of *Lutzomyia* sand flies (Azevedo-Pereira *et al*. 2007). Herein, we have confirmed that these proteins are components of surface membranes and flagella of *L. (V) braziliensis* promastigotes, reinforcing their potential roles in the parasite-vector interaction.

Cell fractionation paired with morphological and/or biochemical methodology has increasingly been used for parasite analyses (de Souza *et al*. 2003). In this study, this approach was applied to investigate HBPs from *L. (V) braziliensis* promastigotes to indicate that cellular membranes and flagella contain two main HBP bands (65 and 55 kDa) after elution from affinity chromatography, as previously described (Azevedo-Pereira *et al*. 2007).

The complex structural organization of heparin receptors has been previously described in other organisms. The mollusk *Anadara granosa* presents an HBP that has a native molecular mass of 300 kDa and is composed of several identical 60 kDa subunits (Dam *et al*. 1994). In *Trypanosoma cruzi*, a 60 kDa HBP was detected in trypomastigote membranes (Ortega-Barria and Pereira, 1991), and these receptors appear to be involved in the interaction of this parasite with cardiomyocytes (Oliveira Jr *et al*. 2008). Based on this evidence, it is possible that the heparin receptor detected in the present study also presents a complex structural organization corresponding to its functionality in *L. (V) braziliensis* promastigotes.

The cell surfaces of *Leishmania* parasites are the first sites of contact between these organisms and different microenvironments; thus, the regulation of surface macromolecule expression provides an important mechanism for adaptation during their life cycle. The surfaces of *Leishmania* spp. have an outer layer consisting of a latticework of carbohydrates covalently linked to lipids and proteins, known as the glycocalyx. This structure is rich in glycoconjugates with complex oligosaccharide structures that may be incorporated into the extracellular matrix or attached to secreted glycoproteins (Novozhilova and Bovin, 2010). Many components of the glycocalyx play roles in the interactions between protozoa and host cells (Chava *et al*. 2004; Naderer *et al*. 2004).

Glycosaminoglycans, such as heparin and heparan sulfate, are covalently attached to core proteins and have different cellular localizations in cells of animal species. Although heparin is found inside mastocytes (Nader *et al*. 1999; Strauss *et al*. 1982), heparan sulfate is found on the cell surface of vertebrates and invertebrates (Cassaro and Dietrich, 1977; Dietrich *et al*. 1980; Nader *et al*. 1984). It is important to note that heparan sulfate is the actual glycosaminoglycan that acts in the interaction between promastigotes and the sand fly gut epithelium; however, because heparin and heparan sulfate are both glycosaminoglycans that share a common chemical structure (Dreyfuss *et al*. 2009), the use of heparin in the binding assays of this study is an acceptable adaptation.

Our analyses show that HBPs from *L. (V.) braziliensis* promastigotes surface have biochemical properties of metallo-proteinases, which are well-known glycoproteins present on the surface and flagellar

pockets of *Leishmania* spp. and other trypanosomatids (Yao, 2010). In recent years, it has been shown that the molecular weights of metallo-proteinases of *Leishmania* spp. are not homogeneous, and enzymes of this class with molecular weights ranging from 50 kDa to values above 63 kDa have previously been detected (Alves *et al*. 2004; Cuervo *et al*. 2006).

For the first time, the interaction of HBPs from *L. (V.) braziliensis* promastigotes with heparin was assessed in real time using SPR. The use of this technology is a new trend in identifying cell surface proteins using a biosensing system (Velasco-Garcia, 2009) due to its flexible and powerful capacity for detecting biomolecular interactions (Tanious *et al*. 2008). SPR analysis enabled the real-time assessment of the interaction between HBPs from the promastigote surface and immobilized heparin (without specific markers). This method was performed in a similar manner to a previously proposed technique for the detection of ligands in mammalian cells (Quinn *et al*. 2000; Hide *et al*. 2002) and pathogenic microorganisms in the environment or in food (Bergwerff and van Knapen, 2006).

Biosensing surface technologies have been useful in elucidating the adhesion and invasion processes in parasite-host interactions that involve parasite proteins and binding with glycosaminoglycans. For example, biosensing surface methods have been used to assess the interactions between heparin and a malarian circumsporozoite protein and demonstrate the role of parasitic glycosaminoglycan receptors in the invasion of liver cells (Rathore *et al*. 2001). In addition, the direct measurement of the binding between the purified measles virus and heparin proved that this interaction prevents the infection of SLAM-negative cells lines (Terao-Muto *et al*. 2008). Therefore, our results that indicated the binding of promastigotes to immobilized heparin onto a biosensing surface confirm the presence of heparin receptors in the surface of the parasites and also support the effectiveness of this methodology.

It was also observed that the HBPs present in flagella are more efficient at binding to heparin than HBPs from the plasma membranes of promastigotes. However, further studies are necessary to clarify the reasons for this difference and to define the nature of the glycosaminoglycan binding site involved in this interaction while considering the quantity of saccharides of the different glycosaminoglycans.

Collectively, our results provide evidence that HBPs, which are the heparin receptors from the surface of *L. (V.) braziliensis* promastigotes, present characteristics of metallo-proteinases and are capable of forming stable complexes with glycosaminoglycans (similar to the activity of heparin). It is possible that HBPs modulate signalling activity in the cellular environment and play specific roles in parasite-host interactions.

ACKNOWLEDGMENTS

We are grateful for the transmission electron microscopy facility of the Oswaldo Cruz Institute. Luzia M. C. Côrtes, M.Sc. is a doctoral student at the Fiocruz institution; Francisco O. R. O. Jr, M.Sc. is a doctoral student at the Fiocruz/CAPES institution, and Franklin S. Silva, M.Sc. is doctoral study fellow at the Fiocruz/CNPq institution. Bernardo A. S. Pereira, Ph.D. is a postdoctoral researcher fellow at the CAPES/FAPERJ. Carlos R. Alves, PhD and Mirian C. S. Pereira, Ph.D. are fellow researchers at the CNPq institution.

FINANCIAL SUPPORT

This work is supported by the Brazilian research agencies PAPES V – CNPq/Fiocruz (300731/2010-8), CNPq (509737/2010-2), CAPES (EDITAL – 11/2009) and FAPERJ (E-26/103.060/2008, E-26/110.257/2010).

REFERENCES

Alves, C. R., Marzochi, M. C. and Giovanni-de-Simone, S. (1993). Heterogeneity of cysteine proteinases in *Leishmania braziliensis* and *Leishmania major*. *Brazilian Journal of Medical and Biological Research* **26**, 167–171.

Alves, J. L., Mendonca-Lima, F. W. and Alves, C. R. (2004). The use of metal chelate affinity chromatography on the isolation of *Leishmania chagasi* promastigote hydrophobic proteinases. *Veterinary Parasitology* **30**, 137–145.

Azevedo-Pereira, R. L., Pereira, M. C. S., Oliveira, F. O. R. Jr., Brazil, R. P., Côrtes, L. M. C., Madeira, M. F., Santos, A. L. S., Toma, L. and Alves, C. R. (2007). Heparin binding proteins from *Leishmania (Viannia) braziliensis* promastigotes. *Veterinary Parasitology* **145**, 234–239.

Bates, P. A. and Rogers, M. E. (2004). New insights into the developmental biology and transmission mechanisms of *Leishmania*. *Current Molecular Medicine* **4**, 601–609.

Becker, I., Salaiza, N., Aguirre, M., Delgado, J., Carrillo-Carrasco, N., Kobeh, L. G., Ruiz, A., Cervantes, R., Torres, A. P., Cabrera, N., González, A., Maldonado, C. and Isibasi, A. (2003). Leishmania lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. *Molecular and Biochemical Parasitology* **130**, 65–74.

Bergwerff, A. A. and van Knapen, F. (2006). Surface plasmon resonance biosensors for detection of pathogenic microorganisms: strategies to secure food and environmental safety. *Journal of AOAC International* **89**, 826–831.

Brandonisio, O., Spinelli, R. and Pepe, M. (2004). Dendritic cells in *Leishmania* infection. *Microbes and infection* **6**, 1402–1409.

Brittingham, A., Morrison, C. J., McMaster, W. R., McGwire, B. S., Chang, K. P. and Mosser, D. M. (1995). Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. *The Journal of Immunology* **155**, 3102–3111.

Butcher, B. A., Sklar, L. A., Seamer, L. C. and Glew, R. H. (1992). Heparin enhances the interaction of infective *Leishmania donovani* promastigotes with mouse peritoneal macrophages. A fluorescence flow cytometric analysis. *Journal of Immunology* **148**, 2879–2886.

Cassaro, C. M. and Dietrich, C. P. (1977). Distribution of sulfated mucopolysaccharides in invertebrates. *The Journal of Biological Chemistry* **252**, 2254–2261.

Chava, A. K., Bandyopadhyay, S., Chatterjee, M. and Mandal, C. (2004). Sialoglycans in protozoal diseases: their detection, modes of acquisition and emerging biological roles. *Glycoconjugate Journal* **20**, 199–206.

Cuervo, P., Saboia-Vahia, L., Costa Silva-Filho, F., Fernandes, O., Cupolillo, E. and dE Jesus, J. B. (2006). A zymographic study of metalloprotease activities in extracts and extracellular secretions of *Leishmania (Viannia) braziliensis* strains. *Parasitology* **132**, 177–185.

Dam, T. K., Bandyopadhyay, P., Sarkar, M., Ghosal, J., Bhattacharya, A. and Choudhury, A. (1994). Purification and partial characterization of a heparin-binding lectin from the marine clam *Anadara granosa*. *Biochemical and Biophysical Research Communication* **203**, 36–45.

de Souza, W. and da Cunha-e- Silva, N. L. (2003). Cell fractionation of parasitic protozoa – a review. *Memórias do Instituto Oswaldo Cruz* **98**, 151–170.

Descoteaux, A. and Turco, S. J. (1999). Glycoconjugates in *Leishmania* infectivity. *Biochemistry and Biophysical Acta* **1455**, 341–352.

Dietrich, C. P., Sampaio, L. O., Montes, D. E., OCA, H. and Nader, H. B. (1980). Role of sulfated mucopolysaccharides in cell recognition and neoplastic transformation. *Anais da Academia Brasileira de Ciências* **52**, 179–186.

Dreyfuss, J. L., Regatieri, C. V., Jarrouge, T. R., Cavalheiro, R. P., Sampaio, L. O. and Nader, H. B. (2009). Heparan sulfate proteoglycans: structure, protein interactions and cell signaling. *Anais da Academia Brasileira de Ciências* **81**, 409–429.

Heussen, C. and Dowdle, E. B. (1980). Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copalymerized substrates. *Analytical Biochemistry* **102**, 196–202.

Hide, M., Tsutsui, T., Sato, H., Nishimura, T., Morimoto, K., Yamnoto, S. and Yoshizato, K. (2002). Real-time analysis of ligand-induced cell surface and intracellular reactions of living mast cells using a surface-plasmon resonance-based biosensor. *Analytical Biochemistry* **302**, 28–37.

Kahl, L. P. and McMahon-Pratt, D. (1987). Structural and antigenic characterization of a species- and promastigote-specific *Leishmania mexicana amazonensis* membrane protein. *The Journal of Immunology* **138**, 1587–1595.

Kamhawi, S. (2006). Phlebotomine sand flies and *Leishmania* parasites: friends or foes? *Trends in Parasitology* **22**, 439–345.

Kock, N. P., Gabius, H. J., Schmitz, J. and Schotteliu, J. (1997). Receptors for carbohydrate ligands including heparin on the cell surface of *Leishmania* and other trypanosomatids. *Tropical Medicine and International Health* **2**, 863–874.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

Lainson, R. and Shaw, J. J. (1987). Evolution, classification and geographical distribution. In *The Leishmaniases in Biology and Medicine* (ed. Peters, W. and Killick-Kendrick, E.), pp. 1–120. Academic Press, London, UK.

Lodge, R. and Descoteaux, A. (2008). *Leishmania* invasion and phagosome biogenesis. *Subcellular Biochemistry* **47**, 174–181.

Love, D. C., Esko, J. D. and Mosser, D. M. (1993). A heparin-binding activity on *Leishmania* amastigotes which mediates adhesion to cellular proteoglycans. *The Journal of Cell Biology* **123**, 759–766.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

McConville, M. J., Homans, S. W., Thomas-Oates, J. E., Dell, A. and Bacic, A. (1990). Structures of the glycoinositolphospholipids from *Leishmania major*. A family of novel galactofuranose-containing glycolipids. *Journal of Biological Chemistry* **265**, 7385–7394.

Moody, S. F. (1993). Molecular variation in *Leishmania*. *Acta Tropica* **53**, 185–204.

Morgado-Diaz, J. A., Silva-Lopez, R. E., Alves, C. R., Soares, M. J., Corte-Real, S. and Giovanni-De-Simoni, S. (2005). Subcellular localization of an intracellular serine protease of 68 kDa in *Leishmania (Leishmania) amazonensis* promastigotes. *Memórias do Instituto Oswaldo Cruz* **100**, 377–383.

Mukhopadhyay, N. K., Shome, K., Saha, A. K., Hassell, J. R. and Glew, R. H. (1989). Heparin binds to *Leishmania donovani* promastigotes and inhibits protein phosphorylation. *The Biochemical Journal* **264**, 517–525.

Murray, P. J., Spithill, T. W. and Handman, E. (1989). The PSA-2 glycoprotein complex of *Leishmania major* is a glycosylphosphatidylinositol-linked promastigote surface antigen. *The Journal of Immunology* **143**, 4221–4226.

Nader, H. B., Ferreira, T. M., Paiva, J. F., Medeiros, M. G., Jeronimo, S. M., Paiva, V. M. and Dietrich, C. P. (1984). Isolation and structural studies of heparan sulfates and chondroitin sulfates from three species of molluscs. *The Journal of Biological Chemistry* **259**, 1431–1435.

Nader, H. B., Chavante, S. F., dos-Santos, E. A., Oliveira, T. W., de-Paiva, J. F., Jerônimo, S. M., Medeiros, G. F., de-Abreu, L. R., Leite, E. L., de-Sousa-Filho, J. F., Castro, R. A., Toma, L., Tersariol, I. L., Porcionatto, M. A. and Dietrich, C. P. (1999). Heparan sulfates and heparins: similar compounds performing the same functions in vertebrates and invertebrates? *Brazilian Journal of Medical and Biological Research* **32**, 529–538.

Naderer, T., Vince, J. E. and McConville, M. J. (2004). Surface determinants of *Leishmania* parasites and their role in infectivity in the mammalian host. *Current Molecular Medicine* **4**, 649–665.

Novozhilova, N. M. and Bovin, N. V. (2010). Structure, functions, and biosynthesis of glycoconjugates of *Leishmania* spp. cell surface. *Biochemistry* **75**, 686–694.

Oliveira, F. O. Jr., Alves, C. R., Calvet, C. M., Toma, L., Bouças, R. I., Nader, H. B., Castro Côrtes, L. M., Krieger, M. A., Meirelles, M. de N. and Souza Pereira, M. C. (2008). *Trypanosoma cruzi* heparin-binding proteins and the nature of the host cell heparan sulfate-binding domain. *Microbial and Pathogenesis* **44**, 329–338.

Ortega-Barria, E. and Pereira, M. E. (1991). A novel *T. cruzi* heparin binding protein promotes fibroblast adhesion and penetration of engineered bacteria and trypanosomes into mammalian cells. *Cell* **67**, 411–421.

Pimenta, P. F., Modi, G. B., Pereira, S. T., Shahabuddin, M. and Sacks, D. L. (1997). A novel role for the peritrophic matrix in protecting *Leishmania* from the hydrolytic activities of the sand fly midgut. *Parasitology* **115**, 359–369.

Quinn, J. G., O'Neill, S., Doyle, A., McAtamney, C., Diamond, D., McCraith, B. D. and O'Kennedy, R. (2000). Development and application of surface Plasmon resonance-based biosensors for the detection of cell-ligand interactions. *Analytical Biochemistry* **261**, 135–143.

Rangel, E. F. and Lainson, R. (2003). Ecologia das leishmanioses. Transmissores de leishmaniose tegumentar Americana. In *Flebotomíneos do Brasil* (ed. Rangel, E. F. and Lainson, R.), pp. 291–309. Fiocruz, Rio de Janeiro, Brazil.

Rathore, D., McCutchan, T. F., Garboczi, D. N., Toida, T., Hernáiz, M. J., LeBrun, L. A., Lang, C. S. and Linhardt, R. J. (2001). Direct measurement of the interactions of glycosaminoglycans and a heparin decasaccharide with the malaria circumsporozoite protein. *Biochemistry* **40**, 11518–11524.

Rebello, K. M., Côrtes, L. M., Pereira, B. A., Pascarelli, B. M., Côrte-Real, S., Finkelstein, L. C., Pinho, R. T., d'Avila-Levy, C. M. and Alves, C. R. (2009). Cysteine proteinases from promastigotes of *Leishmania (Viannia) braziliensis*. *Parasitology Research* **106**, 95–104.

Reithinger, R., Dujardin, J. C., Louzir, H., Pirmez, C., Alexander, B. and Brooker, S. (2007). Cutaneous leishmaniasis. *The Lancet Infectious Diseases* **7**, 581–596.

Sacks, D. and Kamhawi, S. (2001). Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. *Annual Review of Microbiology* **55**, 453–83.

Soares, R. P., Margonari, C., Secundino, N. C., Macedo, M. E., da Costa, S. M., Rangel, E. F., Pimenta, P. F. and Turco, S. J. (2010). Differential midgut attachment of *Leishmania (Viannia) braziliensis* in the sand flies *Lutzomyia (Nyssomyia) whitmani* and *Lutzomyia (Nyssomyia) intermedia*. *Journal of Biomedicine and Biotechnology* **2010**, doi: 10.1155/2010/439174 <http://dx.crossref.org/10.1155%2F2010%2F439174>.

Soares, R. P., Cardoso, T. L., Barron, T., Araújo, M. S., Pimenta, P. F. and Turco, S. J. (2005). *Leishmania braziliensis*: a novel mechanism in the lipophosphoglycan regulation during metacyclogenesis. *International Journal for Parasitology* **35**, 245–253.

Soong, L. (2008). Modulation of dendritic cell function by *Leishmania* parasites. *The Journal of Immunology* **180**, 4355–4360.

Strauss, A. H., Nader, H. B., Takahashi, H. K. and Dietrich, C. P. (1982). Ontogeny of heparin in mammals: a correlation with the appearance of mast cells in tissues. *Anais da Academia Brasileira de Ciências* **54**, 439–448.

Symons, F. M., Murray, P. J., Ji, H., Simpson, R. J., Osborn, A. H. and Cappai, R. (1994). Characterization of a polymorphic family of integral membrane proteins in promastigotes of different *Leishmania* species. *Molecular and Biochemical Parasitology* **67**, 103–113.

Tanious, F. A., Nguyen, B. and Wilson, W. D. (2008). Biosensor-surface plasmon resonance methods for quantitative analysis of biomolecular interactions. *Methods in Cell Biology* **84**, 53–77.

Terao-Muto, Y., Yoneda, M., Seki, T., Watanabe, A., Tsukiyama-Kohara, K., Fujita, K. and Kai, C. (2008). Heparin-like glycosaminoglycans prevent the infection of measles virus in SLAM-negative cell lines. *Antiviral Research* **80**, 370–376.

Velasco-Garcia, M. N. (2009). Optical biosensors for probing at the cellular level: a review of recent progress and future prospects. *Seminars in Cell & Developmental Biology* **20**, 27–33.

Volf, P., Svobodova, M. and Dvorakova, E. (2001). Bloodmeal digestion, *Leishmania major* infections in *Phlebotomus duboscqi*: effect of carbohydrates inhibiting midgut lectin activity. *Medicine Veterinary Entomology* **15**, 281–286.

Yao, C. (2010). Major surface protease of trypanosomatids: one size fits all? *Infection and Immunity* **78**, 22–31.