Research Article

Journal of PeptideScience

Received: 14 April 2011 Revised: 27 May 2011 Accepted: 10 June 2011 Published online in Wiley Online Library: 1 August 2011

(wileyonlinelibrary.com) DOI 10.1002/psc.1392

Dermaseptin 01 as antimicrobial peptide with rich biotechnological potential: study of peptide interaction with membranes containing *Leishmania amazonensis* lipid-rich extract and membrane models

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This article addresses the interactions of the synthetic antimicrobial peptide dermaseptin 01 (GLWSTIKQKGKEAAIAAA-KAAGQAALGAL-NH_2, DS 01) with phospholipid (PL) monolayers comprising (i) a lipid-rich extract of *Leishmania amazonensis* (LRE-La), (ii) zwitterionic PL (dipalmitoylphosphatidylcholine, DPPC), and (iii) negatively charged PL (dipalmitoylphosphatidylglycerol, DPPG). The degree of interaction of DS 01 with the different biomembrane models was quantified from equilibrium and dynamic liquid-air interface parameters. At low peptide concentrations, interactions between DS 01 and zwitterionic PL, as well as with the LRE-La monolayers were very weak, whereas with negatively charged PLs the interactions were stronger. For peptide concentrations above 1 μg/ml, a considerable expansion of negatively charged monolayers occurred. In the case of DPPC, it was possible to return to the original lipid area in the condensed phase, suggesting that the peptide was expelled from the monolayer. However, in the case of DPPG, the average area per lipid molecule in the presence of DS 01 was higher than pure PLs even at high surface pressures, suggesting that at least part of DS 01 remained incorporated in the monolayer. For the LRE-La monolayers, DS 01 also remained in the monolayer. This is the first report on the antiparasitic activity of AMPs using Langmuir monolayers of a natural lipid extract from *L. amazonensis*.

Keywords: dermaseptin 01; antimicrobial peptides; *Leishmania amazonensis*; model membranes; phospholipid monolayers; dilatational surface elasticity; nanomedicine for neglected diseases

Introduction

During the last two decades, an increasing number of antimicrobial peptides (AMPs) have been discovered in animals and plants, as well as in bacteria [1,2]. Due to the increasing resistance of pathogenic organisms to conventional antibiotics, research on antimicrobial agents including peptides has been highly relevant and intense [3]. Conventional antibiotics can induce more resistance in microbial cells than the AMPs [4]. The biological activities of AMPs are believed to be strongly related to their interactions with the cell membranes [5–8]. Due to the high complexity of the biological cell membranes, one usually employs biomimetic model systems to investigate the effects of composition and physical properties of the phospholipid (PL) matrix on the membrane properties. These models include planar lipid bilayers, multilamellar and unilamellar vesicles, lipid microspheres, micelles, and Langmuir monolayers [9–13].

Dermaseptins (DSs) represent a family of AMPs isolated from the skin secretion of frogs from *Phyllomedusa* genus [14,15]. Their chemical structure comprises cationic molecules containing 28–34 amino acids that fold into amphiphilic helices in contact with hydrophobic media. The DS polypeptide chains are gene encoded

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as part of larger precursor molecules comprising a signal peptide with 22 residues, followed by an acidic propeptide, a typical prohormone processing signal, and a DS progenitor sequence. DSs show cytolytic action against numerous microorganisms. They have been considered promising agents to fight viral diseases [16,17], drug resistant bacteria [18,19], protozoa [14,19], yeasts, and filamentous fungi [15,20,21]. It has been demonstrated that these peptides do not demonstrate significant cytolysis against mammalian blood cells [22].

DS 01 (GLWSTIKQKGKEAAIAAAKAAGQAALGAL-NH_2), a DS collected from the skin of *Phyllomedusa oreades* [19] and *Phyllomedusa hypochondrialis* frogs, has demonstrated highly antibacterial activity against Gram-negative and Gram-positive bacteria as well as anti-protozoan activity against *Trypanosoma cruzi* (for *in vitro* tests). The activity of DS 01 against *Leishmania amazonensis* in the promastigote form has also been demonstrated recently [14].

Langmuir PL monolayers are advantageous for studying interactions between bioactive molecules and lipid membranes, as there are no curvature effects and the lateral packing can be precisely controlled [23–30]. When incorporated into monolayers, interacting compounds are able to change their physical characteristics, such as packing density, ordering of the film-forming molecules, and electrical properties [28]. In some cases, even the precise location of the active molecule can be determined. Many biologically active compounds, including protein and peptides, have been studied using Langmuir lipid films as model membranes [10–13,28,31–37], despite many of them can interact with both leaflets of a biomembrane.

In this article, we employ the Langmuir monolayers to study the interactions of DS 01 peptide with a lipid-rich extract from *L. amazonensis* (LRE-La) and PL monolayers. To our knowledge, similar studies have not been reported. The present results should contribute to the understanding of the molecular interactions and mechanism of action of this important AMP in biological and lipid membranes. As AMPs can act in timescale of minutes, dynamic studies represented by dynamic dilatational surface elasticity data are included in this work.

Materials and Methods

Lipids

1,2-dipalmitoyl-*sn*-3-glycero-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (DPPG) were purchased from Avanti Polar Lipids, and used as received. Chloroform and methanol were HPLC grade. Lipid-rich extract from *L. amazonensis* (LRE-La), detergent resistant membrane (DRM) were obtained as described in Santos *et al*. [13]. The pellet (DRM) obtained after ultracentrifugation (100 000 *g* for 1 h at 4 °C) of the 0.1% (w/v) SDS solubilized membrane fraction was resuspended in water and treated with equal volume of chloroform and methanol (1 : 1 v/v). Water phase was discarded and the lipid-rich solvent phase that corresponded to the *Leishmania* membranes, constituted by native extract of *L. amazonensis,* was stored at −20 °C and used in all experiments.

Antimicrobial Peptide Synthesis

The peptide DS 01 was synthesized by a solid phase method and purified as previously described [19,38]. The amidated DS 01 was synthesized on a Pioneer Synthesis System from Applied Biosystems (Framingham, MA, USA). Fmoc-amino acids and Fmoc PAL-PEG-PS resin were purchased from Applied Biosystems. Purification of the peptide required a preparative C_{18} column (Vydac 218 TP 1022) on a HPLC system Class LC-10VP. Molecular mass (2793.6 Da) and sample purity was checked by MALDI-TOF MS. The final purification step of this synthetic peptide was performed by RP-HPLC on a Vydac 218 TP 54 analytical column.

Langmuir Monolayers

Surface pressure measurements were carried out on a KSV Langmuir mini-trough mounted in a class 10 000 clean room. Langmuir monolayers were prepared by spreading 25 µl of a 1 mM chloroform or chloroform/methanol PL solutions on the surface of an aqueous subphase. The aqueous subphase consisted of ultra pure water with resistivity of 18 MΩ.cm and a surface tension of 72.4 mN m^{-1}, supplied by a Milli-Ro coupled to a Milli-Q system (MilliPore). After spreading, 10 min were allowed for solvent evaporation and monolayer to reach equilibrium. The peptide solution was injected into the subphase underneath pre-assembled lipid monolayers and its adsorption into the monolayer was monitored.

The surface pressure (π) was determined using the Wilhelmy plate method. Compressing speed was maintained at 10 mm/min. All the experiments were performed at 22 ± 1 °C.

Dilatational Surface Elasticity

The dynamic elasticity for PL monolayers formed on pure water and on DS 01 aqueous solution was determined at 30 ± 2 mN m^{-1} (corresponding to pressure of several biomembranes) [39,40]. It was measured by the deformation of pendant drops and the axisymmetric drop shape analysis method (OCA-20, Dataphysics Instruments GmbH, Germany), with oscillating drop accessory ODG-20, as described in the literature [41,42]. In this method, a PL chloroform solution of *ca*. 10^{-4} mol l^{-1} is gently touched on the surface of a reduced size drop, formed by pure water or different DS 01 concentrations aqueous solutions. The drop was then rapidly expanded up to a predetermined drop area rendering the desired surface pressure. The dynamic surface elasticity data were obtained after the surface tension reached a constant value by using a periodic drop oscillator with an amplitude of 0.1 mm (relative area variation $\Delta A/A = 5.5\%$) and a frequency of 1.0 Hz. The viscous effect, related to the imaginary part of the elasticity modulus, was estimated from the phase angle.

Results

Interaction of DS 01 with Langmuir Monolayers

Characterization of peptide insertion into the membrane film relies either on monitoring the time-dependent changes of surface pressure while keeping the film area constant, or keeping the surface pressure constant and recording the changes in molecular surface area as the amphiphilic peptide diffuses into the membrane film [30].

The ability of the peptide to interact with monolayers was initially investigated by measuring the increase in surface pressure ($\Delta\pi$) at a constant molecular area of 105 $Å^2$, upon the injection of the peptide beneath the monolayer film. The adsorption kinetics for DS 01 on the LRE-La monolayers and on the PL monolayer constituted by DPPC and DPPG are shown in Figure 1(A)–(C),

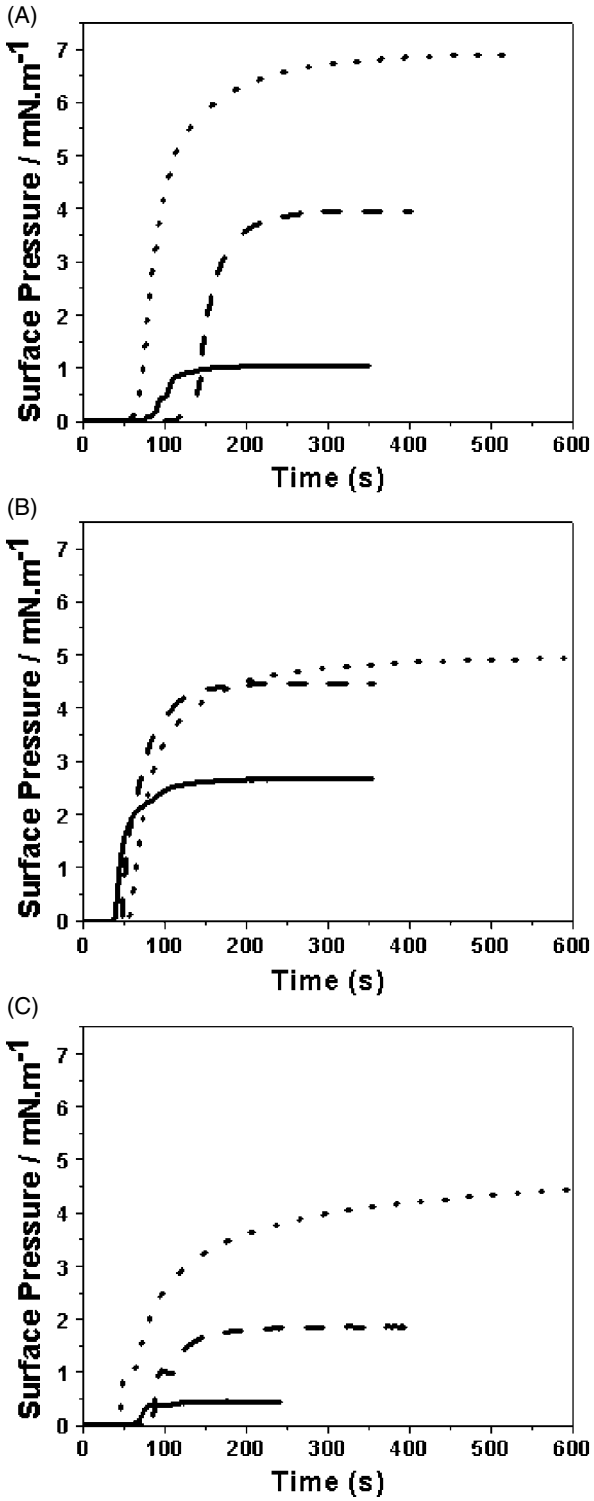

Figure 1. Time-dependence adsorption of DS 01 peptide to: (A) LRE-La, (B) DPPC and (C) DPPG monolayers at air–water interface, monitored by surface pressure measurements. From bottom to top, the following concentrations (μg/ml) of DS 01 were used: 1 (———), 2 (- - - -), 4 (· · · ·).

respectively. In all cases, upon peptide injection, one may observe a sharp increase in surface pressure followed by a plateau.

The higher the peptide concentration, the higher was the equilibrium surface pressure at the plateau.

For LRE-La monolayers, the initial increase in the surface pressure was not dependent on DS 01. Interestingly, this is the same behavior obtained for DPPG monolayers. Conversely, for DPPC monolayers, a dependence of the time the surface pressure starts increasing on peptide concentration was observed.

Incorporation of DS 01 modifies the structure of Langmuir monolayers, as manifested by noticeable changes of pressure-area (π-A) isotherms. Figure 2 shows π-A isotherms for the LRE-La Langmuir monolayers in the absence and presence of DS01, at various peptide concentrations. The solid curve in Figure 2 corresponds to π-A isotherm for the LRE-La membrane and have been reproducibly obtained. Upon addition of DS 01 at 1 μg/ml in the subphase, an expansion to higher molecular areas was observed. The monolayer expansion was higher for higher peptide concentrations. Both liquid-expanded (LE) and liquid-condensed (LC) phases are shifted to larger areas, indicating the presence of the peptide incorporated at the interface, even at high lipid packing.

Figure 3(A) shows π-A isotherms for the zwitterionic DPPC PL monolayers in the absence and in the presence of DS 01, at various peptide concentrations. The solid curve in Figure 3(A) corresponds to the pure PL and is essentially the same reported in previous articles [36,43,44]. Changes were visible from a minimum DS 01 concentration of 1 μg/ml, above which expansion of the monolayers increased with increasing peptide concentration. LE and LC phases are distinguished by the compressional moduli [$C_s^{-1} = -A(\partial\pi/\partial A)_T$]. Therefore, $C_s^{-1} > 100$ mN m^{-1}, corresponds to LC and $C_s^{-1} < 100$ mN m^{-1} to LE [43,44] and both are observed for DPPC. At higher DS 01 concentrations, both LE and LC phases are shifted to higher areas.

The π-A isotherms for the negatively charged DPPG PL monolayers in the absence and in the presence of several concentrations of DS01 are depicted in Figure 3(B). The solid curves correspond to the pure PL, and are essentially the same observed in previous reports [43,44]. The effect of incorporation of DS01 on negatively charged DPPG monolayers was more pronounced than on the LRE-La monolayers and also larger than that exerted on zwitterionic monolayers (Figs 2, 3(A) and (B), respectively). An increase in the DS 01 concentration induced a shift toward larger areas per lipid molecule. Changes are also observed in the LC region, in which the area for close packing increased upon increasing peptide concentration. It is also seen that the latter effect is more pronounced for DPPG than for LRE-La or DPPC.

In DPPC monolayers at higher surface pressures, the area per lipid molecule tends to return to the value observed for pure lipid, probably because DS01 molecules are expelled from the lipid monolayer at high pressures. However, in the case of DPPG, especially at concentrations higher than 2 μg/ml of DS01, the area per lipid molecule in the presence of DS01 remains higher than that for the pure lipid, even at high surface pressures. Electrostatic interactions with the negatively charged interface from DPPG monolayer contributed to prevent the loss of peptide to the subphase. For LRE-La membranes, it was not observed loss of peptide to subphase, and again the presence of a net negative charge may also prevent the loss of the peptide due to electrostatic interactions [45].

Interaction of DS01 with Phospholipid Monolayers by Dilatational Surface Elasticity Measurements

The incorporation of the peptide in PL monolayers, at a lipid packing correspondent to a natural biomembrane was performed by the pendant drop technique, performing dilatational surface

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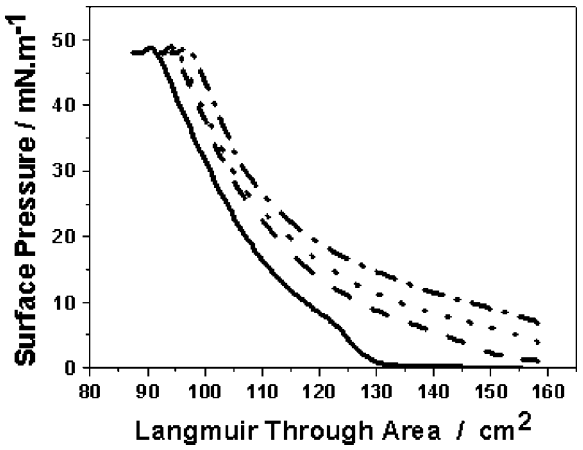

Figure 2. Surface pressure isotherms of LRE-La monolayers in the presence of variable DS01 final concentrations (μg/ml): 0 (———), 1 (- - - -), 2 (· · · ·), 4 (—. — . — .—).

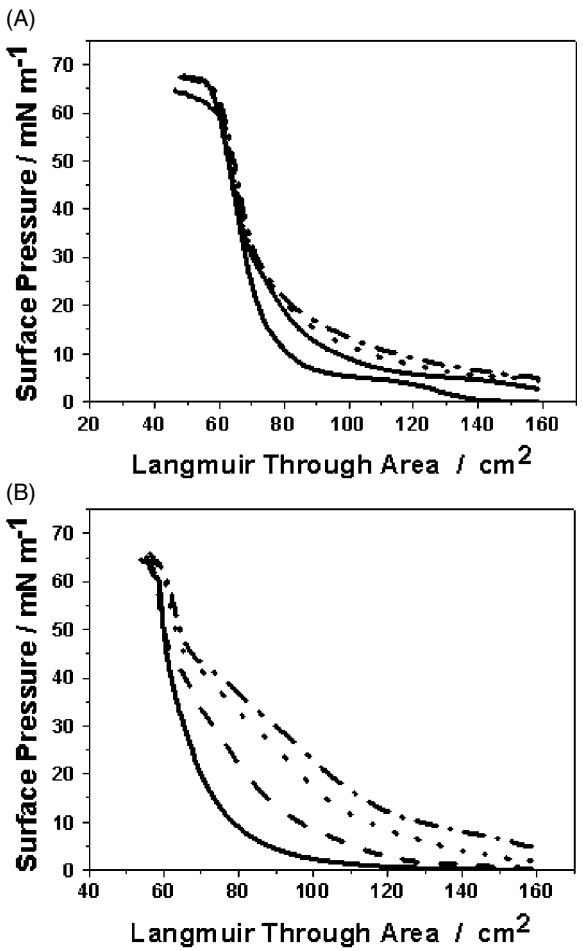

Figure 3. Surface pressure isotherms of (A) DPPC and (B) DPPG PL monolayers in the presence of variable DS01 concentrations (μg/ml): 0 (———), 1 (- - - -), 2 (· · · ·), 4 (—. — . — .—).

elasticity measurements as described in the Experimental Section. The results are presented in Figure 4(A) for DPPC monolayers, and Figure 4(B) for DPPG monolayers. For DPPC monolayers, an increase in the peptide concentration caused a slightly reduction in

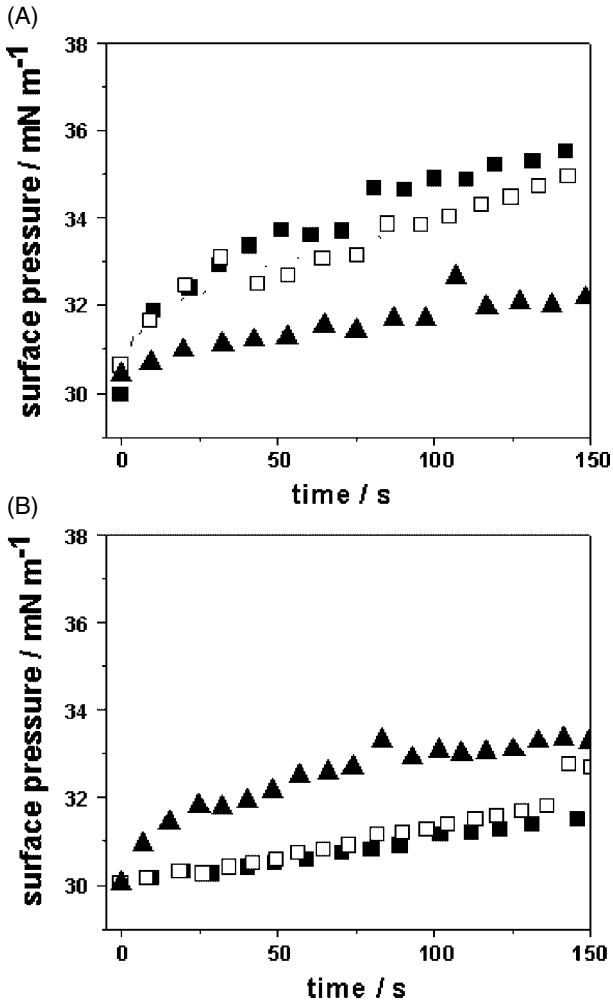

Figure 4. Adsorption kinetics, from surface pressure variations, for DS 01 on (A) DPPC and (B) DPPG monolayers, at initial surface pressure of 30 mN m^{-1}. DS 01 concentrations (μg/ml): 1 (■), 2 (□), 4 (▲).

the rate of surface pressure increase, in contrast to the equilibrium data, represented by the π-*A* isotherms observed in Figure 3(A), and also to the adsorption kinetics at low surface packing, as presented in Figure 1(A)–(C). It is important to note that for both conditions the peptide was incorporated to the monolayer at low lipid packing. Besides, at high lipid packing DS 01 was expelled from the interface. In the case of the adsorption kinetics measured with the pendant drop technique, however, we measure the ability of the peptide, present in a homogeneous solution, to interact with a preformed lipid monolayer initially at high compression (30 mN m^{-1}). These results indicate that DS 01 exhibits low affinity for a stable, already-formed DPPC monolayer. For DPPG monolayers, however, a different behavior may be seen, i.e. surface pressure increases upon increasing peptide concentration. This can be attributed to an efficient adsorption of the peptide on the negatively charged monolayer, being this effect more pronounced for higher DS 01 concentrations.

Periodic surface deformation for obtaining the dilatational surface elasticity modulus (*E*) was applied after allowing *ca.* 200 s for the peptide adsorption. Note that the curves for DPPG (Figure 4(B)) did not reach constant values. Nevertheless, upon deformation we did not observe significant drift in the surface

Table 1. Elastic modulus (*E*) for different surface pressures of DPPG and DPPC monolayers after interaction with different concentrations of DS01

	DPPG		DPPC	
DS01 (μg/ml)	$\pi_{200\ s} \pm 0.2$ (mN m^{-1})	*E* (mN m^{-1})	$\pi_{150s} \pm 0.2$ (mN m^{-1})	*E* (mN m^{-1})
0	–	232.3		219.7
1	32.1	131.9	35.5	209.23
2	32.8	147.5	34.6	224.3
4	33.4	158.8	32.2	241.4

pressure values. The resultant surface elasticity data indicated how the presence of the peptide affected the mixed monolayer. The *E* values are presented in Table 1.

Discussion

The time dependence of the DS 01 adsorption on PL monolayers, DPPC and DPPG, and on LRE-La monolayers showed a nonlinear kinetics behavior, with the surface pressure reaching a plateau in a time scale of minutes. To a first approximation, the time-dependence of the surface pressure increases as a function of peptide concentration following a simple Langmuir adsorption isotherm. Within the concentration range investigated, the binding of DS 01 follows a single exponential behavior. The pressure rise until its steady-state values and these values depend on peptide concentration. The higher the peptide concentration, the higher the surface pressure values at the steady-state. The differences observed for the surface pressure steady-state values can be accounted for the different composition in each monolayer system.

Incorporation of DS 01 affected both the LRE-La and the PL monolayers, which become more expanded. This effect, which started at concentrations of 1 μg/ml DS 01, was higher for DPPG, in comparison to LRE-La (Figures 2 and 3(B)), or to DPPC (Figure 3(A) and (B)). For higher concentrations, the interaction between DS 01 and DPPC was higher than with the negatively charged DPPG. Despite the higher surface pressure variation for DPPC, the system appears to be close to the saturation, as a further increase in the DS 01 concentration did not corresponded to a significant increase in the surface pressure.

DS 01 is more effective in increasing the surface pressure of LRE-La monolayer at concentrations higher than 2 μg/ml. For DPPC a limiting concentration effect is observed. In all cases there is a time lag for surface pressure increase upon injection. This time is concentration dependent for LRE-La and the anionic PL DPPG, being practically invariant for DPPC (*ca.* 50 s). Both LRE-La and DPPG exhibited the smallest times for the highest DS 01 concentration, 4 μg/ml, as expected for a simple diffusion mechanism from the bulk to the interface. However, the highest time lag is observed for the intermediary concentration of DS 01.

In the condensed phase, small changes were observed for both lipids. The fact that DS 01 binds to zwitterionic PLs – albeit to a lesser extent than to negatively charged ones – points to the contribution of interactions other than electrostatic for binding. The presence of a more hydrophobic region in the middle of the peptide would be responsible for the ability of DS 01 to bind to the zwitterionic PLs.

In the case of negatively charged DPPG, binding of positively charged DS 01 is favored by electrostatic interactions, leading to dramatic changes in the lateral packing of this monolayer (Figure 3(B)). The surface pressure *versus* molecular area isotherms for DPPC and DPPG monolayers clearly express the preference of DS 01 for net negatively charged lipids over zwitterionic ones. When considering the interaction of DS 01 with LRE-La monolayers, the surface pressure *versus* through area shows that for low peptide concentration (1 μg/ml) the peptide-monolayer interaction is the lowest. However, for high peptide concentration (4 μg/ml) the degree of peptide-membrane interaction is similar to that one observed of DPPC monolayers. While DPPC and DPPG monolayers are composed of pure PLs, LRE-La membranes contain different PLs, being the membrane composition much more complex. *L. amazonensis* membranes have a multicomponent nature, containing lipids, glicolipids, and proteins [46,47]. The composition of PLs and sphingolipids in *Leishmania* is quite different from that in mammals, which may reflect the difference in membrane physiology between parasites and hosts. As major membrane components, these lipids may determine the membrane permeability and fluidity and have profound impact in vesicular trafficking, nutrient acquisition through endocytosis, or cell differentiation which involves extensive membrane remodeling/reorganization and macroautophagy. In addition, the polyunsaturated fatty acid chains found in phosphatidylcholine (PC) could confer resistance to host-derived oxidants [48]. Overall, PLs account for about 70% of total cellular lipids in *Leishmania* [48–50]. Major PLs species classified according to the 'headgroup' include PC (30–40%), phosphatidylethanolamine (PE, about 10%), and phosphatidylinositol (about 10%) [50–52]. Despite the intrinsic complexity of the membrane, information on the surface membrane chemistry of *Leishmania* is useful to understand the behavior observed with DS 01. It has been demonstrated that *Leishmania*'s membrane possesses a net negative surface charge [46–48] due to its constituents, including PLs and carbohydrates [46,47]. Based upon surface pressure measurements, we may predict about the interaction of the peptide with LRE-La membrane. The results may corroborate biological tests that showed the action of DS 01 against *L. amazonensis* in the promastigote form [14]. The presence of sterols in LRE-La membranes [53] may account for the lower interactions between DS 01 and the LRE-La monolayers due to the higher molecular packing promoted by sterols when compared with the same type of interaction with DPPC PLs with lower peptide concentration, or with DPPG PLs at any peptide concentration.

The effect of DS 01 on DPPC and DPPG monolayers was also investigated by dilatational surface elasticity measurements. For an insoluble one-component system this technique provides information on how easily the system recovers a surface tension value, after a perturbation. The higher the dilatational elasticity modulus, *E*, the more difficult is to change the surface packing by compression–expansion sequences. In the case of mixed monolayers (insoluble/insoluble or soluble/insoluble components) reaction or interaction at the interface should be considered, that can also be reflected in the dissipative component. In this study, the dissipative component was very low (less than 5 mN m^{-1} for both DPPC and DPPG at any DS 01 concentration). Once again, one could observe differences of the molecular interaction of DS 01 with DPPC and DPPG monolayers. For DPPG, at a DS 01 concentration of 1 μg/ml the *E* value decreased sharply when compared to the *E* value for the pure monolayer. The latter was interpreted as the incorporation of the peptide into the lipid monolayer, promoting a large perturbation, and consequently making the film to lose its original rigidity. The increase in the

elasticity upon increasing the peptide concentration reveals that DS 01 accumulates in the interface, rendering the mixed film (peptide + lipid) rigid again. Similar behavior has been observed before for the peptide Pln 149 [54].

For DPPC monolayer, a quite distinct behavior was noticed. Despite adsorption kinetics showed a variation with surface pressure (suggesting the interaction of DS 01 with the monolayer), dilatational surface elasticity measurements showed that the peptide exerts small influence on the monolayer structure, which was evinced by the small changes of *E* values, not surpassing 10%. Such result indicates that probably there is small penetration of the peptide in the monolayer.

The results are also in agreement with those from Silva *et al*. [55] regarding the interaction between DS 01 with membrane-mimetic environment and model lipid membranes. In that case, circular dichroism studies revealed the ability of DS 01 to adopt an α-helices conformation in a membrane-mimetic environment, and fluorescence studies showed binding of DS 01 to zwitterionic large unilamellar vesicles (LUV). Furthermore, atomic force microscopy showed that DS 01 peptide was able to induce morphological changes in zwitterionic LUV. To our knowledge, there are no reports on the interaction of DS 01 with negatively charged lipids so far. The markedly larger effects of DS 01 on the charged PL DPPG in our monolayer studies indicate that the changes caused by the peptide are primarily due to a stronger binding promoted by electrostatic interactions between the positively charged peptide and the negatively charged PL headgroups. The preference of DS 01 for negatively charged PL monolayers is consistent with results obtained for other AMPs [31,32,56–61]. In addition, the behavior of DS 01 toward membrane interactions is in agreement with other types of DSs that also showed a preference to negatively charged lipids over zwitterionic lipids [62].

Several AMPs showed the ability to form ion channel in lipid bilayers with the ion channel activity depending on lipid composition [63–65]. Dermaspetins also showed the ability to form ion channels in zwitterionic and negatively charged planar lipid bilayers, with the ion channel activity being more prominent with the latter type of bilayer [66]. It might be possible that DS 01 displays a similar behavior due to the common features shared with other DSs, including (i) their linear structure with 28–34 amino acid residues; (ii) cationic character, due to the high content of lysine residues; (iii) ability to form α-helices in a membrane-mimetic environment.

The results obtained here also corroborate the findings that AMPs have stronger effect on bacterial membranes in comparison to mammalian cell membranes [67–69]. Despite the high complexity of bacterial cell membranes, there is well-documented information concerning the PL composition of membranes of Gram-negative and Gram-positive bacteria [67,69]. A common feature in both cases is the presence of high amounts of PLs carrying negatively charged headgroups, such as phosphatidylglycerol (PG), cardiolipin, and the zwitterionic phospholipid PE [70,71]. In contrast, the outer leaflet of mammalian cell membranes comprises mainly uncharged PC, sphingomyelin, and cholesterol. In this latter case, most of the lipids with negatively charged headgroups are segregated in the inner leaflet, facing the cytoplasm [70,71].

Conclusions

The data obtained in this study using equilibrium and dynamics measurements of the AMP DS 01 with Langmuir monolayers as a biomembrane model provide evidence at the molecular level for the preference of the AMP DS 01 for negatively charged membranes (when excluding other composition parameters). And, more important, the obtained results indicate that the AMP DS 01 promotes rather significant structural changes in the membrane composed by PG compared with phosphatidylcholine, as attested by the elasticity moduli data. We could attest that low concentrations of DS 01 decreases the surface packing of the membrane model, whereas larger amounts accumulate at the interface increasing the rigidity of the interface. In addition, this is the first report on the investigation of antiparasitic activity of AMPs using Langmuir monolayers of a natural lipid extract from *L. amazonensis,* which has reinforced our results with simpler models of prepared lipid mixtures. The results may be useful for possible applications involving encapsulation of DS 01 for subsequent administration/delivery, resulting in increased affinity and selectivity.

Acknowledgements

This work is supported by CNPq (Universal 2009 and Consolidação Novos Campi 2009), Nanobiomed Capes Network (Nanobiotec/CAPES/Brasil), Fundação de Apoio à Pesquisa do Estado do Piauí (FAPEPI process 20203.0608/2008 Fluxo Contínuo), and FAPESP (Brazil). L.C.S. was a CNPq post-doctoral fellow; T.M.N. is a FAPESP post-doctoral fellow.

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