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Abstract

Lipophosphoglycan (LPG) is a lypopolysaccharide found on the surface of the parasite *Leishmania donovani* that is thought to play an essential role in the infection of humans with leishamniasis. LPG acts as an adhesion point for the parasite to the gut of the sand fly, whose bite is responsible for transmitting the disease. In addition, LPG acts to inhibit protein kinase C (PKC) in the human macrophage, possibly by structural changes in the membrane. The Ca^{2+} ion is believed to play a role in the infection cycle, acting both as a crosslinker between LPG molecules and by playing a part in modulating PKC activity. To gain insight into the structure of LPG within a supported lipid membrane and into the structural changes that occur due to Ca^{2+} ions, we have employed the atomic force microscope (AFM). We have observed that the LPG molecules inhibit bilayer fusion, resulting in bilayer islands on the mica surface. One experiment suggests that the LPG molecules are parallel to the mica surface and that the structure of the LPG changes upon addition of Ca^{2+}, with an increase in the height of the LPG molecules from the bilayer surface and an almost complete coverage of LPG on the bilayer island.

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Introduction

Leishmania donovani is the parasite that causes human leishmaniasis. There are approximately 1.5 million new cases of leishmaniasis each year and it can be found in about 90 countries.[1] This disease is spread through the bite of a sand fly.[2] When a person is bitten by an infected sand fly the parasite, in the promastigote form, is taken up by macrophages. Inside the macrophage, the parasite transforms into the amastigote form, which avoids destruction from the macrophages and replicates. Ingesting the infected macrophages through another bite then infects a second sand fly. Inside the gut of the sand fly, the parasite transforms back to promastigote form, attaches to the lining of the gut and multiplies. After several days the parasite detaches from the gut and is ready for the next bite.

Lipophosphoglycan (LPG) is the predominant molecule found on the surface of the promastigote form of the parasite. It is therefore believed that LPG plays a major role in the infection cycle and in surviving the harsh environments encountered during this cycle. For example, LPG is thought to control the binding and release of the parasite from the gut of the sand fly. In addition, it has been shown that LPG inhibits protein kinase C (PKC) in the macrophage, allowing the parasite to enter and survive.[3] The binding of Ca^{2+} or other divalent metal ions to the LPG containing membrane may also play a role in the survival of the parasite, most likely due to physical changes within the membrane.

Modeling studies have surmised that LPG exists as either a helical or double helical structure.[4,5] The structure of LPG within the membrane, however, has not been determined experimentally. Atomic force microscopy (AFM) has proven to be a useful tool in the imaging of lipid membrane systems.[6,7,8] In addition, AFM has been used to investigate the mechanical properties of polysaccharides.[9,10,11,12] We report herein the AFM imaging of LPG within a lipid membrane to examine LPG structure at the membrane surface and changes that might occur upon the addition of Ca^{2+} ions.

Experimental

Liposome Preparation. The lipids 1-palmitoyl, 2-oleoyl phosphatidylserine (POPS) and 1-palmitoyl, 2-oleoyl phosphatidylcholine (POPC) were purchased from Avanti Polar Lipids (Alabaster, AL). The liposomes were prepared by dissolving an equimolar mixture of POPS and POPC in a 2:1 chloroform / methanol solution at a total lipid concentration of approximately 1.5 mM. The solution was evaporated to a thin film on the walls of a glass centrifuge tube using a rotary evaporator. Residual solvent was removed from the film by further drying overnight under high vacuum. The film was then hydrated in 3.0 mL of Tris buffer solution (0.02 M Tris, 5 mM $MgCl_2$ and 0.1 M KCl in purified 18 MΩ water, pH 7.0) at 65˚C with vortex stirring, producing a total lipid concentration of 3.5 mM. The completely suspended film was first degassed with N_2 gas for several minutes, then sonicated with a 3 mm probe tip at 25W power under N_2 gas. Sonication was performed in 4 minute cycles with 1 minute resting between each cycle for a total of 20 minutes at room temperature. The translucent solution was centrifuged for 20 minutes at 15,000g to remove large bilayer aggregates. The clear supernatant was filtered through a 0.2 micron filter. The liposomes containing lipophosphoglycan (LPG) were prepared similarly, except the dried film was hydrated with 2 ml of Tris buffer solution and, after probe sonication, 1 ml of an LPG solution (1 mg / ml in Tris buffer, pH 7.0) was added. This solution was then bath sonicated for 10 minutes prior to centrifuging. Dilution of LPG to the desired concentration in the membrane was achieved by varying the amount of LPG solution added to the POPS / POPC bilayers.

Atomic Force Microscopy. AFM experiments were performed with a Nanoscope IIIa Multimode scanning probe microscope (Digital Instruments, Santa Barbara, CA). The images were acquired in tapping mode in solution using a commercially available liquid cell (Digital Instruments) with 120-µm oxide-sharpened silicon nitride V-shaped cantilevers. The nominal spring constant of the cantilever was 0.35 N/m. Images were collected with the J scanner, which has a maximum range of 120

um x 120 um, operating at a scan rate of 2 Hz. The images were collected with 256 data points per line.

The supported lipid bilayers were prepared via vesicle fusion on a freshly cleaved mica substrate. The clean mica was first imaged in Tris buffer to establish the baseline. The liposome solution was then injected into the AFM liquid cell and the mica surface imaged subsequently. The liposome solution was incubated with the mica at room temperature for approximately one hour to allow optimal time for vesicle fusion.

Results and Discussion

Bilayer Formation. Previously, liposomes of 1:1 POPS / POPC containing LPG were prepared and the inhibition of PKC studied.[3] As a direct comparison to these studies, we also used POPS / POPC liposomes. Liposomes containing 50 mol% POPS / POPC readily form continuous lipid bilayers on a freshly cleaved mica surface by vesicle fusion. The liposomes are fluid phase at room temperature and, under typical conditions, no holes are observed in the bilayers when imaged with AFM. Therefore, it can be difficult to distinguish between the bilayer and the bare mica surface. To prove the existence of a bilayer, the liposome solution was diluted in Tris buffer to a lipid concentration of 10 μM and fused to the mica surface to yield partial bilayer coverage on mica. Bilayer disks on the mica surface, which occur before the formation of a continuous bilayer, could then be observed and the measured heights of the disks, 48 ± 4 Å, are consistent with that expected for a lipid bilayer with an 8 –10 Å water layer between the mica and the bilayer (Figure 1a). A 1 mM solution of 50% POPS / POPC added to the AFM fluid cell produces full bilayer coverage immediately on the mica surface (Figure 1b).

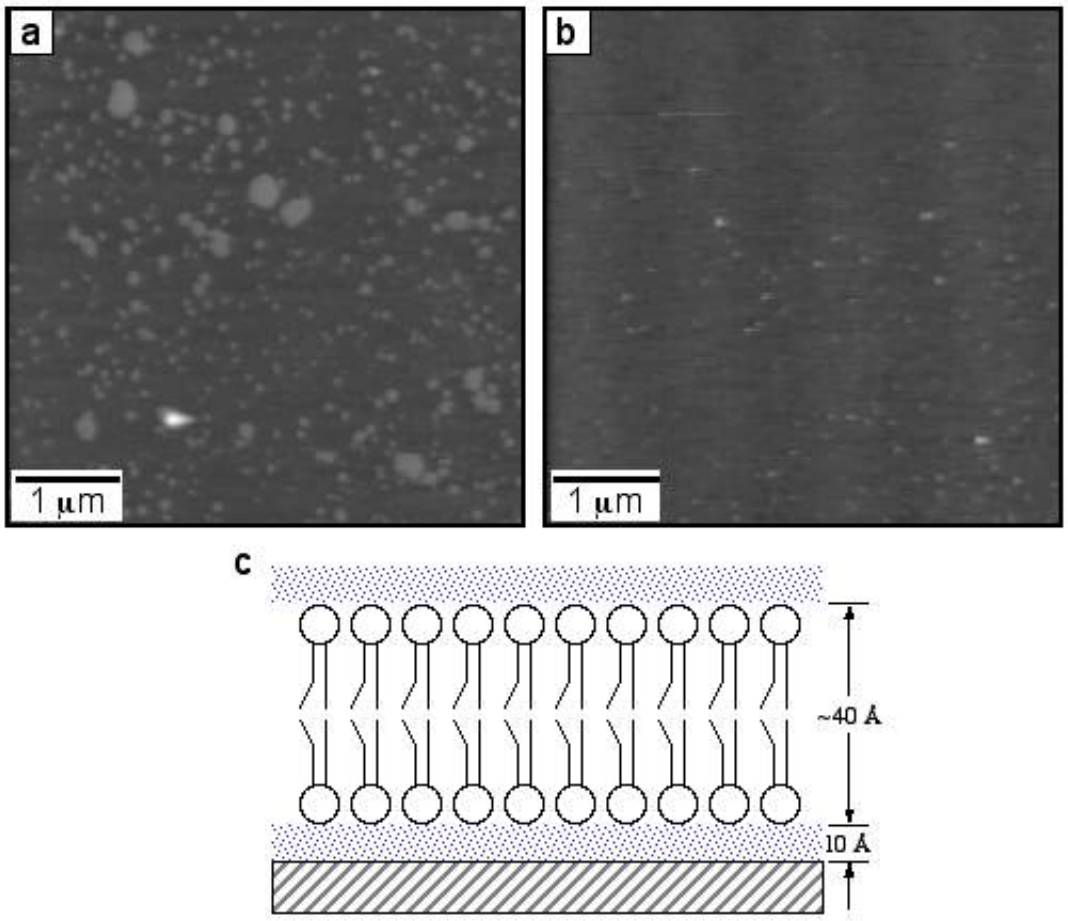

Figure 1. AFM tapping mode images in solution of a 1:1 POPS / POPC bilayer. (a) Bilayer islands formed with a lipid concentration of 10 µM. Height of islands: 48 ± 4Å. (b) A continuous bilayer formed upon the addition of a solution with a lipid concentration of 1mM. (c) Schematic representation of a POPS / POPC bilayer with a 10 Å water layer on a mica substrate.

Liposomes containing varying concentrations of LPG (0.008 mM to 0.04 mM or 1 LPG molecule / 125 lipid molecules to 1 LPG molecule / 25 lipid molecules) were spread as lipid bilayers on a mica surface by vesicle fusion. These liposomes formed bilayer islands but were never observed to form a continuous bilayer (Figure 2). Decreasing the amount of LPG in the liposomes resulted in the formation of larger lipid bilayer islands, indicating that the LPG disrupts bilayer fusion on the mica surface and that the extent of the disruption is concentration dependent. In most cases, the LPG molecules could not be identified in these islands. The exact locations of the LPG are difficult to ascertain in these images and may position themselves at the edges of the bilayer islands, making them difficult to resolve.

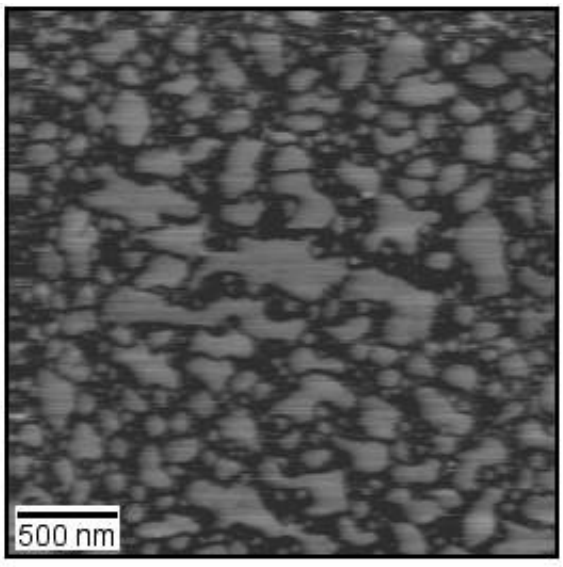

Figure 2. AFM tapping mode image in solution of a 1:1 POPS / POPC bilayer containing 0.04 mM LPG. Bilayers containing LPG did not form a continuous bilayer membrane structure.

Although the LPG molecules were difficult to observe on the membrane surface, one set of experiments produced images that were highly suggestive of LPG in the lipid bilayer islands (Figure 3a). They are visualized as small areas of lighter gray within the islands. The height of the islands relative to the mica surface is 29 ± 3 Å. This height is smaller than that expected for a typical lipid bilayer and that observed for a POPS / POPC bilayer without LPG, indicating that the lipid molecules are either more disordered or have a greater tilt angle when LPG is incorporated. The lighter gray regions on top of the islands have a height of approximately 6.5 ± 2 Å taller than the islands. Based on these heights, we have assigned the darkest regions to the mica surface, the medium gray regions to the POPS / POPC lipid bilayer, and the small light gray regions to the LPG molecules. The small heights of the LPG above the lipid bilayer suggest that the LPG molecules are lying parallel along the bilayer surface.

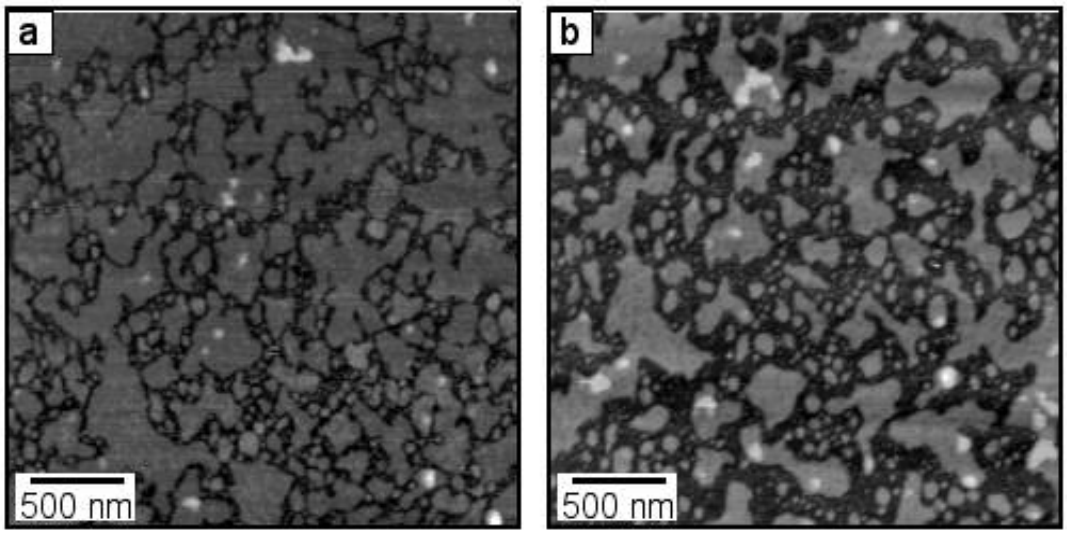

Figure 3. (a) Tapping mode AFM image of a 1:1 POPS / POPC bilayer containing 0.02 mM LPG. (b) Tapping mode AFM image of a 1:1 POPS / POPC bilayer containing 0.04 mM LPG and 40 mM $CaCl_2$.

Effect of Ca^{2+}. The Ca^{2+} ion is thought to play an important role in the structure and function of LPG. It is known to act as a crosslinker between LPG molecules, directly affecting the integrity of the parasite. In addition, the binding of Ca^{2+} to LPG may be involved in modulating PKC activity, which is important for infection by Leishmania.

In AFM imaging, there was no observable effect when Ca^{2+} was added to POPS / POPC bilayers that did not contain LPG. However, when Ca^{2+} was added to the POPS / POPC / LPG bilayers, mixed results were obtained. When 10 mM $CaCl_2$ was added before vesicle fusion to liposome solutions containing either 0.04 mM or 0.008 mM LPG, the size of the bilayer islands was found to increase relative to those observed for either LPG concentration without Ca^{2+}. This result is consistent with published reports stating that the presence of Ca^{2+} aids in vesicle fusion.[13] Addition of Ca^{2+} following the formation of bilayer islands on the mica surface, however, resulted in no change in size of the islands in most experiments. The exception to this observation occurred in the experiment in which the LPG molecules were thought to be observed. In this case, the addition of 40 mM $CaCl_2$ resulted in an immediate change in the structure of the bilayer island and the structures attributed to LPG molecules (Figure 3b). The LPG regions now cover almost the entire bilayer island and appear to be 1 – 2 Å taller. The bilayer islands, however, have decreased their surface area. The increase in height of the LPG molecules

suggests that the Ca^{2+} is crosslinking the molecules, resulting in a structural change that is more perpendicular to the lipid bilayer surface.

Effect of LPG Antibody. To verify the presence of LPG in the bilayer, the IgM antibody was used. The antibody has a molecular weight of 700 kDa and should be easily observable with the AFM. As a control, the antibody was added to a supported bilayer containing only POPS and POPC to confirm the lack of interaction of the antibody with the bilayer. The POPS/POPC bilayer was prepared by vesicle fusion and no defects were observed. Upon addition of the antibody, however, structural changes were observed in the bilayer. Immediately, the bilayer appeared to be rough in spots and a closer examination revealed that the bilayer partially dissolved (Figure 4a). Moving the AFM tip to other locations on the bilayer showed additional areas of dissolved bilayer that do not cover the entire scanning area, confirming that the AFM tip is not causing the dissolution.

Also observed on the bilayer are small islands that were not seen before addition of the antibody (Figure 4b). These islands have an approximate height of 25Å and are assumed to be lipids from the dissolved areas depositing on top of the membrane. Unfortunately, the difficulties encountered with the antibody prohibited us from identifying LPG within the membrane.

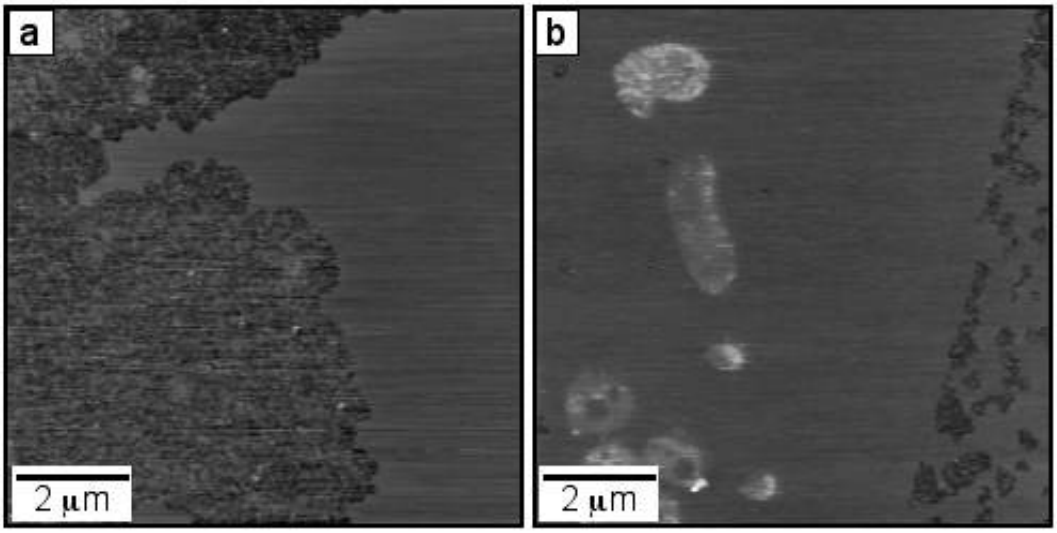

Figure 4. Tapping mode AFM images taken after addition of a LPG antibody to a POPS / POPC bilayer showing (a) partial bilayer dissolution and (b) partial bilayer dissolution as well as additional islands on the membrane surface. The islands have an approximate height of 25 Å.

Conclusions

Although liposomes containing 50 mol% POPS / POPC formed continuous lipid bilayers on a mica surface, the addition of LPG causes the formation of bilayer islands that do not coalesce to form a full coverage bilayer. In addition, the size of the islands decreased with increasing LPG concentration suggesting that the addition of LPG inhibits bilayer fusion on the mica surface. While LPG did influence the bilayer structure, it was not readily observed in the islands with AFM. One experiment did reveal structures within the islands that appear to be LPG lying parallel to the mica surface. Also, the structure of the LPG was affected by the addition of Ca^{2+} ions, with the LPG covering most of the bilayer island. While these experiments have yielded some insight into the structure of LPG within a supported lipid bilayer, more experiments are needed to assess the repeatability of these observations.

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