Aggregation of Gramicidin A in Phospholipid Langmuir–Blodgett Monolayers

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ABSTRACT The aggregation of Gramicidin A (gA) in dipalmitoylphosphatidylcoline (DPPC) monolayers is investigated by both thermodynamic and structural methods. Compression isotherm analysis and atomic force microscopy (AFM) observations are performed. Our experimental results indicate that gA aggregation does occur in DPPC monolayers even at very low gA concentration (about 8×10^{-4} mol%). At the low gA concentration limit, the aggregation process seems to be mainly horizontal (i.e., side-by-side, into the monolayer plane), following a fractal pattern growth producing the formation of typical, flat (0.5 nm height) "doughnut" structures, with a diameter of ~150 nm. These structures appear to be composed of smaller subunits (about 70 nm diameter) showing the same doughnut structure. At a molar fraction of ~3.8 mol%, the big doughnuts start to disaggregate and only small doughnuts appear. Above a gA concentration of ~4.4 mol%, all doughnuts (large and small) disappear, and the morphology assumes the appearance of a patchwork of two distinct phases: one that, being very flat, can be associated with a gA-free or gA-poor DPPC phase, and a second one, characterized by a more corrugated surface, associated with a gA-rich DPPC phase. At gA concentration of ~5 mol%, a percolation transition in the gA-rich DPPC phase occurs. Thermodynamic data indicate that the maximum of miscibility between gA and DPPC molecules occurs at ~28 mol%, suggesting that gA could aggregate in hexamers that are, on average, bound to 16 DPPC molecules. At the same concentration, AFM images show a network of small gA aggregation units of a size compatible with gA hexamers.

INTRODUCTION

Cell membranes are structures that play a key role in cell physiology and are responsible for a number of important functions such as ionic transport, receptor recognition, signal transduction, etc. The molecular structure of this important and complex system is closely related to the specific function involved. The composition and physical state of the phospholipids and proteins constituting biological membranes are known to vary according to the leaflet (outer or inner) and the cell site (surface, cytoplasm, or nucleus). Moreover, many membrane proteins do not work as isolated units, and their lateral aggregation is a crucial process by which they perform biological functions. In fact, lateral aggregation has been demonstrated to modulate the phase structure of biomembranes (Killian and de Kruijff, 1985).

Up to now, the statement that "changes in the conformation of a protein invariably produce an effect on its biological activity" sounds quite obvious. Understanding the complex interplay of the different interactions among lipid matrix, proteins, and cytoskeleton in determining membrane activity represents the new frontier of membrane science. In this sense, the study of both protein aggregation in a model membrane mimicking the lipid matrix, and the influence of different physico-chemical parameters on this process, is of great relevance to the understanding of the basic mechanisms that govern membrane function.

Gramicidin A (gA) is a small peptide of 15 amino acids, forming the smallest known transmembrane ion channel with a high degree of specificity for monovalent cations. Urry (1971) proposed, on the basis of solution nuclear magnetic resonance technique, that the structure of gA membrane channel is a dimer of β-helices disposed head-to-head relative to each other. Subsequent nuclear magnetic resonance studies (Ketchem et al., 1993) established that the helices are right-handed. A considerable body of work confirms the idea that the channel structure in a lipid bilayer is the proposed head-to-head dimer of two right-handed single-stranded β-helices (HHSH), each monomer spanning one half of the double layer (Andersen et al., 1999). A controversy still remains because there is an alternative structure that could provide a pore large enough to transport ions: the right-handed double-stranded double-helical (DSDH) dimer structure, spanning the whole membrane (Burkart et al., 1998; Burkart and Duax, 1999). This is the form that crystallizes out of organic solvents (Langs, 1998). However, a general consensus seems to be in favor of the HHSH motif as the dominant one in membranes. This is probably due to the tendency of the tryptophan residues of the gA to be disposed in the membrane region where the phospholipid headgroups and the electrolytes interpenetrate each other. This view was recently supported by molecular dynamics simulations performed to complement fundamental nuclear magnetic resonance investigations on the effect of gA on the lipid structure at various concentrations (Rice and Oldfield, 1979). Molecular dynamics simulation pro-

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vides information about bonding between the gA channel (HHSH) and surrounding lipids, in the presence of water molecules. Both Woolf's simulation (Woolf and Roux 1996), concerning a gA dimer with 16 lipids (gA-lipid ratio of 1/8) and Chiu's simulation (Chiu et al., 1999), performed in excess of lipids, suggest that the gA channel is surrounded by 16 lipids. It is worth noting that Chiu's simulation provides additional information about the thickness of the lipid bilayer near the gA molecules. They concluded that the thickness is greater immediately adjacent to the channel than it is farther away.

As noted above, the study of protein aggregation as a function of the concentration would help to understand the molecular mechanisms managing the biological events associated with membrane proteins. Up to now, there has not been general agreement among published results concerning gA aggregation. Morrow and Davis (1988) reported the coexistence of two phases (gel and liquid crystal) for gA in dipalmitoylphosphatidylcholine (DPPC) bilayers, at molar fractions below 2 mol%. Above this fraction, a continuous phase was found, suggesting that gA could be uniformly distributed. However, Killian and de Kruijff (1985) proposed that gA is highly aggregated in DPPC phospholipid bilayers in the gel state only above 6 mol%, whereas He et al. (1993) reported that, at least for dilauroylphosphatidylcholine bilayers, gA is uniformly distributed up to 10 mol%. Interestingly, Spisni et al. (1983), on the basis of freeze–fracture electron microscopy, fluorescence, and circular dichroism measurements, described the formation of elongated gA aggregations in lisophosphatidylcholine bilayers at a molar fraction of ~10 mol% and proposed an aggregation model based on "basic units of gA hexamers." More recently, Mou et al. (1996) applied atomic force microscopy (AFM) to the investigation of supported PC bilayers in the gel state, confirming the aggregation based on "basic hexameric units" proposed by Spisni (1983). They also investigated the aggregation process as a function of gA concentration and observed a "percolation transition" at ~5 mol%, in agreement with the aggregation onset at ~6 mol%, previously described by Killian and de Kruijff (1985).

Because of the intrinsic complexity of such living systems as biomembranes, the choice of a simplified model membrane, where all experimental parameters can be accurately monitored, is of great importance in the study of the elemental processes of protein–lipid interaction. Phospholipid monolayers, prepared by the Langmuir–Blodgett (L-B) technique, are best known as a simplified model system of biological membranes (Roberts, 1990). The Langmuir technique allows a monolayer of selected or mixed phospholipids to be prepared at the water–air interface. The investigation of phase behavior and molecular organization of lipid monolayers has recently been reviewed by Maget-Dana (1999) as a powerful tool for the study of interfacial properties of a wide family of membrane-lytic peptides, such as gA. Studies performed on monolayers complement those performed on bilayers. Moreover, because the gA single-stranded β-helix (SH) vertical size (2.5 nm) is comparable to that of DPPC monolayer (3 nm), the effects of the hydrophobic mismatch in the complex interactions occurring between peptide and lipids are reduced (Mobashery et al., 1997). Moreover, the study of gA aggregation in L-B monolayers provides a unique opportunity for the thermodynamic and structural characterization of the peptide–lipid system. In fact, the L-B trough allows for both compression isotherm analysis of a monolayer and its deposition onto a solid substrate, at a defined surface pressure.

Lipid monolayers deposited onto solid substrates have been widely used to study the structure and properties of native biological membranes and to investigate biological processes such as molecular recognition, enzymatic catalysis, cell adhesion, and membrane fusion (for a recent and complete review, see Dufrêne, 2000). In contrast, a variety of applications relying on supported lipid films have been developed (such as biosensors). Moreover, many surface spectroscopic techniques are available to study the structure, composition, and properties of supported lipid monolayers. Until recently, however, due to a lack of high-resolution surface-imaging techniques, little was known about the structure and properties of lipid films at nanometer level.

AFM has now opened up exciting new possibilities in this area. The technique allows study of the lipid surface nanostructure and measurement of surface physical properties, even for individual molecules in air, vacuum, and aqueous environments. Different imaging modes have been developed: contact-mode and tapping-mode AFM, also defined as intermittent contact-mode, where the probe is excited externally and the amplitude and phase of the cantilever are monitored near the resonance frequency. The drawback to contact-mode is that tip-sample interaction forces can damage soft biological samples. In contrast, tapping-mode AFM is suitable for such samples because probe-sample lateral forces are greatly reduced. However, AFM is limited by many factors influencing the image obtained. Sample-tip forces can be introduced by hydrophobic interaction occurring between the tip and different zones of the surface, often due to differences in water accumulation. Another important factor is the broadening and distortion effect introduced by the finite size and shape of the tip (tip-locus effect). Despite these factors, however, the AFM technique is able to obtain molecular lateral resolution and subnanometer vertical resolution, yielding molecular-scale topographic maps of surfaces.

The aim of this work is to investigate, in a membrane model constituted by DPPC monolayers deposited at the air–water interface, the aggregation process of gA molecules as a function of their concentration. The influence of gA molecules on the DPPC physical state is studied by measuring the isotherm changes, and a simple interpretation model is proposed. Finally, monolayers are deposited onto

mica and structurally characterized by a new noncontact AFM, called needle-sensor AFM, able to obtain images with lateral resolution similar to that obtained in contact-mode, without the disadvantages of the tip-to-sample touching.

METHODS

Langmuir–Blodgett films

Mixed gA/DPPC monolayers with increasing gA concentration were prepared at the air–water interface following the Langmuir technique (Roberts, 1990; Bordi et al., 1999). gA and DPPC were purchased from Sigma (St. Louis, MO) with a purity of more than 99.9%.

Mixed lipid-gA solutions were prepared in chloroform (1 mg ml^{-1}) at different molar fractions of about 0, 8×10^{-4}, 3×10^{-2}, 8×10^{-2}, 3, 3.8, 4.4, 5, 10, 28, 50, 80, 100 mol% of gA/DPPC. Appropriate amounts of solution (24 μl) were spread with a microsyringe onto the aqueous subphase. To allow sufficient solvent evaporation, monolayers at the air–subphase interface were rested ~10 min before compression. All experiments were carried out on a subphase of 10 mM KCl, 1 mM Hepes (pH = 7.2), thermostatted by a water-circulating bath at a temperature of 25.0 ± 0.2°C.

The surface tension-area isotherms were obtained by means of a computer-controlled commercial device (Minitrough, KSV, Helsinki, Finland) enclosed in a Plexiglas box to reduce surface contamination. Symmetric compression was achieved with two moving barriers at a constant rate of 10 mm min^{-1}. The surface tension (π) of the lipid monolayer was measured using the Wilhelmy method, using a roughened platinum plate, with an accuracy of 1 mN m^{-1}.

Monolayers were deposited onto freshly cleaved mica substrate by vertically extracting the mica sheet through the film at a constant rate of 0.1 mm min^{-1} and keeping constant the film surface tension at 35 mN m^{-1}. The mica had been previously half-dipped into the subphase before monolayer deposition. Langmuir–Blodgett monolayers are stable over a long period of time if stored in a dry atmosphere. Films relative to gA concentration of 8×10^{-4}, 8×10^{-2}, 3.8, 4.4, 5, and 28 mol% were studied at molecular resolution by AFM. In the case of 8×10^{-2} mol%, the monolayer was deposited at two surface pressures, 35 and 10 mN m^{-1}.

Atomic force microscopy

Experiments were performed in air using a noncontact AFM, based on a needle-sensor AFM from Omicron (Taunusstein, Germany). The probe consists of a long, thin needle cemented onto the front of a quartz rod oscillated by a 1-MHz generator (Bartzke et al., 1993). The needle, which oscillates with a magnitude of a few nanometers and at a distance of 10 nanometers from the sample, is influenced by the gradient of either lateral or vertical forces. As a result, resonance parameters such as amplitude, frequency, and phase shift of the quartz oscillator may change, and discrimination of phase shift between the generator and the oscillator provides the required signal for AFM. The needle-sensor AFM operates like a tapping-mode AFM. Because of the vertical oscillation, it experiences gradient of attractive and repulsive forces but without real contact with the sample surface. In the constant phase-shift mode, the tip is moved back and forth to maintain a constant phase-shift value, and the resulting image provides the topography of the sample (z).

Because of the tip radius (about 10 nm) and water deposited onto its surface, the lateral dimensions of features have to be convoluted with the so-called "tip-locus-effect." We have tested x, y dimensions with Au colloidals particles with a claimed diameter of 10 nm, and we have found an additive broadening of ~25 nm. To investigate the influence of the water layer, in vacuum (5×10^{-10} mbar) measurements were performed in the same instrument and on the same samples previously observed in air. We did not obtain any significant difference.

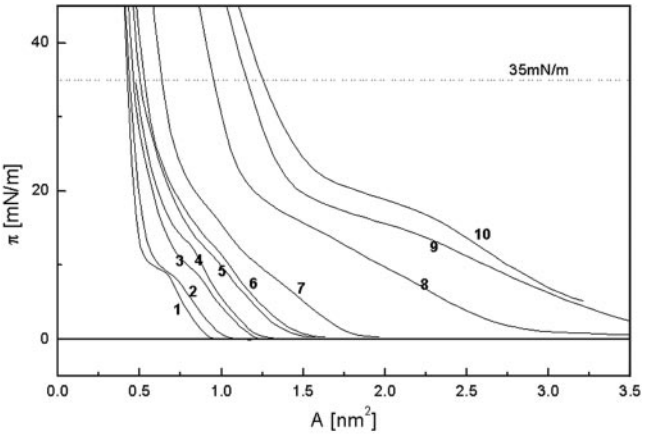

FIGURE 1 L-B compression isotherms of gA-DPPC mixed films and pure component. Curves correspond to gA concentrations, in mol%, of (*1*) 0, (*2*) 3×10^{-2}, (*3*) 3, (*4*) 3.8, (*5*) 5, (*6*) 10, (*7*) 28, (*8*) 50, (*9*) 80, (*10*) 100, respectively.

RESULTS AND DISCUSSION

Thermodynamic data

The main parameters that characterize the monolayer are the temperature (T), the surface pressure (π), the surface area, and the number of molecules. These last two parameters are expressed as area per molecule (A), defined as the total area of the surface between the mobile barriers divided by the total number of molecules deposited at the interface (when different molecular species are present at the surface, A assumes the meaning of mean area per molecule).

Typical compression isotherms of pure Langmuir films (DPPC, *curve 1* and gA, *curve 10*) and mixed films (gA-DPPC at increasing gA concentration, *curves 2–9*), are reported in Fig. 1. A typical DPPC pressure-area ($\pi - A$) isotherm at $T = 25$°C (Fig. 1, *curve 1*) shows characteristic features: a gaseous phase for A greater than 0.9 nm^2; a liquid-expanded phase for $0.7 < A < 0.9$ nm^2; a plateau region between 0.5 and 0.7 nm^2, corresponding to a lipid phase transition from liquid-expanded to liquid-condensed; below 0.5 nm^2, the step increase of the curve indicates the onset of a liquid-condensed phase; eventually, at A values lower than 0.4 nm^2, a solid phase is reached.

Figure 1 shows that, even at very low concentration, the presence of gA molecules strongly affects the curve shape. As the gA concentration increases, the curves are gradually shifted toward higher A values, and the typical plateau shown by the DPPC isotherm gradually disappears.

Isotherms up to 3 mol% are quite similar to each other in shape, whereas isotherms for 3.8, 5, and 10 mol% (*curves 4*, *5*, and *6*, respectively) are characterized by a different shape, with a smooth plateau at higher π and A values. The curve relative to 28 mol% (*curve 7*) shows a quite different shape, with the plateau typical of the gA and DPPC isotherms reduced to a barely appreciable change in slope.

Finally, the curves for 50 and 80 mol% (*curves 8* and *9*) are very similar to the pure gA isotherm. The excess free energy of mixing, ΔG, for the isotherm of a mixed monolayer can be defined as (Maget-Dana, 1999):

$$\Delta G = \int_0^{\pi} A_{12}\,\mathrm{d}\pi - X_1\int_0^{\pi} A_1\,\mathrm{d}\pi - X_2\int_0^{\pi} A_2\,\mathrm{d}\pi, \quad (1)$$

where subscripts 1 and 2 refer to DPPC and gA, respectively, and subscript 12 to the mixture. X represents the molar fraction $X = [\mathrm{gA}]/([\mathrm{gA}] + [\mathrm{DPPC}])$. The lower limit is generally zero, whereas the upper limit can be chosen on the basis of different considerations. It is generally accepted that monolayers mimic bilayer behavior at surface pressures between 30 and 50 mN m^{-1} (Jones and Chapman, 1995). An upper limit of $\pi = 35$ mN m^{-1} was therefore chosen to calculate the excess free energy of the mixture, and all L-B films (if not specifically indicated) were deposited onto mica at this surface-tension value.

Excess free energy ΔG, resulting from differences between the areas under the mixed isotherm and the weighted average of single-component isotherms, provides information on whether a particular interaction is energetically favored ($\Delta G < 0$) or not ($\Delta G > 0$) compared to an ideal mixture, where it is assumed that no difference exists in the interaction potentials when a molecule interacts with another molecule of the same molecular species or with a different one. Roughly speaking, values of $\Delta G > 0$ can be interpreted as a tendency of the molecules to interact preferentially with molecules of the same kind, suggesting that at least one component could form aggregates. Moreover, a minimum in the plot of ΔG versus molar fraction usually indicates the formation of a complex between molecules of different species.

The excess free energy (ΔG) for all the gA-DPPC mixtures at different molar ratios investigated, up to a surface pressure of 35 mN m^{-1}, was calculated according to Eq. 1. The results are shown in Fig. 2 *A*. This analysis indicates that there was a strong immiscibility up to 5 mol% with the formation of gA clusters in the dominant lipid phase. Similarly, a strong immiscibility was evident at gA concentration greater than about 50 mol% with, in this case, the formation of DPPC clusters in the dominant protein phase. Between 5 and 50 mol%, ΔG shows a minimum, and at 28 mol% is close to zero, suggesting that, at this concentration, the behavior of mixed films becomes more and more ideal, with the two molecular species completely mixed.

The mean molecular area A measured at $\pi = 35$ mN m^{-1} for the gA-DPPC mixtures at all the different molar ratios investigated is shown in Fig. 2 *B*. For ideal mixtures, the mean molecular area of mixed monolayers can be calculated as an average of the areas per molecule measured at the same pressure for pure monolayers, using the molar fractions as weights. Therefore, in the ideal case, the mean molecular area A shows

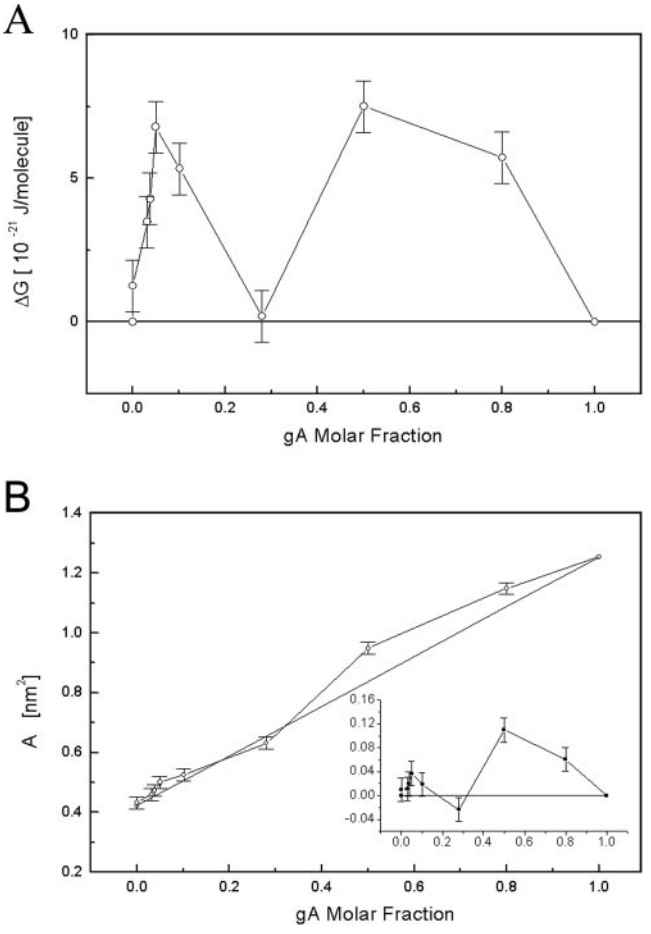

FIGURE 2 (*A*) Energy analysis relative to spectra of Fig. 1. The excess free energy of mixed monolayers measured at surface tension $\pi = 35$ mN m^{-1} ΔG versus gA molar fraction X. (*B*) Area analysis, the mean area per molecule A versus the gA molar fraction X. Inset details deviations from linearity.

a linear behavior as a function of the mole ratio. The inset of Fig. 2 *B* shows the deviations from linear behavior that we observed. For molar fractions lower than 5 mol% and higher than 50 mol%, positive deviation with respect to the linear behavior suggests the formation of bidimensional clusters. At molar fractions ~28 mol%, a minimum is observed. Our results indicate that, at ~28 mol%, the system is in a state of maximum miscibility in which a gA-DPPC complex should occur. Considering the molar fraction at which the minimum occurs, the complex should be formed between 28 gA and 72 DPPC molecules.

As outlined in the introduction, molecular dynamics simulations described the formation of gA–lipid complexes at a molecular ratio of 1/8. Moreover, the existence of a "basic aggregation unit" made of gA hexamer has been proposed. As sketched in Fig. 5 *A*, the upper view of a gA monomer SH is triangular, whereas, for a hexamer (Fig. 5 *B*) it is hexagonal (Spisni et al., 1983, Mou et al., 1996). Assuming that eight lipids are bound to the three sides of the monomer, it is

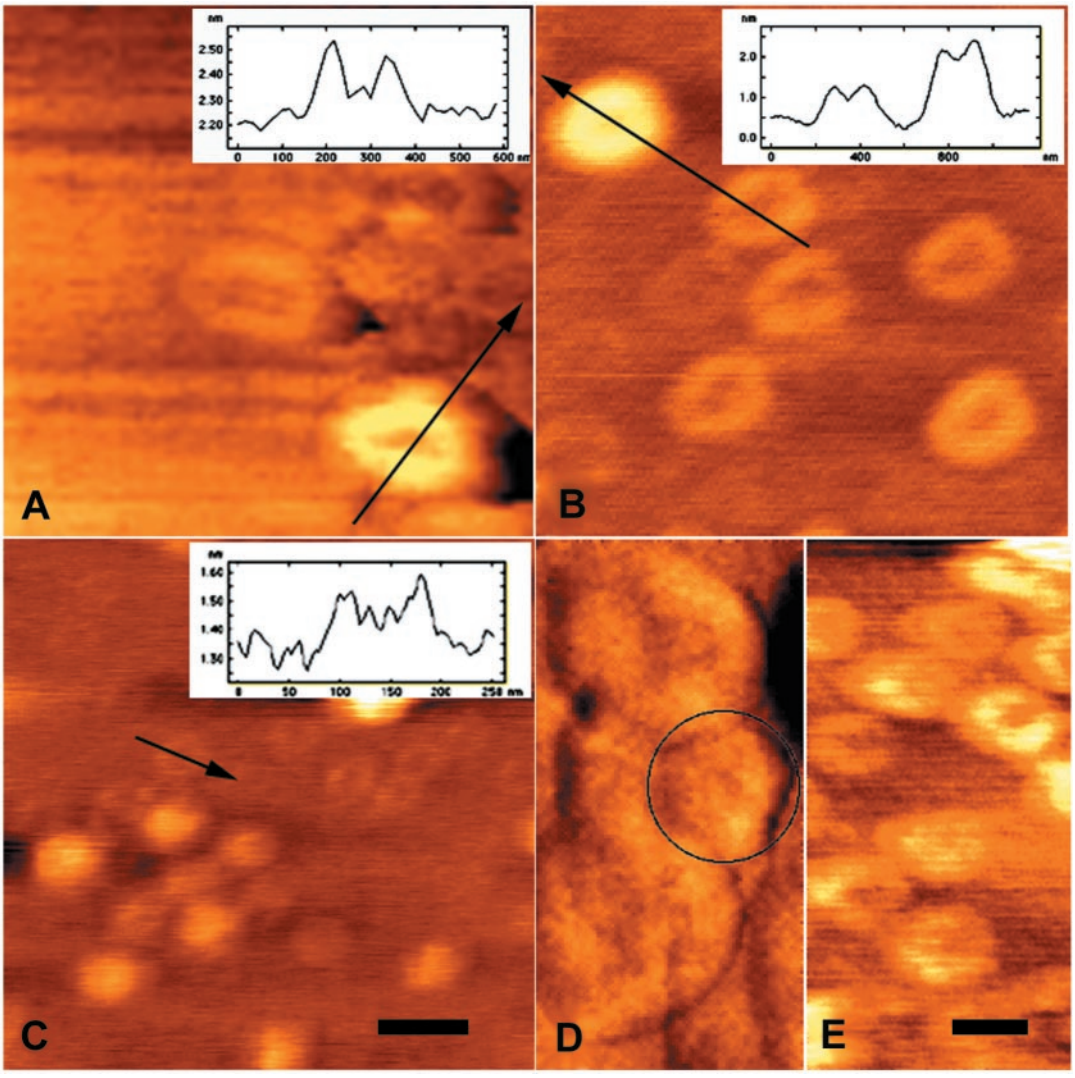

FIGURE 3 AFM images relative to gA-DPPC samples of gA concentration of (*A*) 8×10^{-4}, (*B*) 8×10^{-2}, and (*C*) 3.8 mol% (bar represents 200 nm). The arrows indicate the direction of line profiles reported in the insets. (*D* and *E*) Magnifications of (*B*) (bar represents 50 nm). In particular, (*D*) shows doughnuts of ~70 nm (*circled*) constituting the largest doughnuts of ~150 nm. (*E*) Doughnuts of ~70 nm, not yet aggregated.

possible to suppose that 16 lipids are bound to the six sides of the hexamer. The complex hexamer-lipids could therefore occur at a molecular ratio of 6/16, very close to the molecular ratio at which we obtained the maximum of miscibility (28/72). Based on this consideration, we confirm that the smallest aggregation unit of gA in DPPC is a hexamer of SH.

Horizontal growth

AFM images reported in Figs. 3 and 4 are selected from many images for six gA concentrations. Monolayers were deposited at a surface pressure of 35 mN m^{-1}. Figure 3 shows three molar fractions (8×10^{-4}, 8×10^{-2}, 3.8 mol%) representative of the concentration range in which thermodynamic data suggest the formation of gA clusters. AFM observations of mixed gA-DPPC samples of 8×10^{-4} and 8×10^{-2} mol% (Fig. 3, *A* and *B*, respectively) revealed a typical structure characterized by a rounded doughnut shape. More than 30 doughnuts were measured, and a normal distribution of the dimensions was observed, probably due to different tip-curvature radius or humidity conditions. Mean values of ~150 ± 30 nm in external diameter, and of ~70 ± 10 nm for the hole in the center, can be calculated.

The density of doughnut structures depended on gA concentration. The images show selected fields of samples rich in these typical structures. The ratio between the white (gA aggregates) and dark areas (DPPC) of images reported in Fig. 3 is not representative of the actual gA-DPPC molar ratio. As can be expected, samples with low protein concentration (<3.8 mol%) showed large areas of DPPC layer, free of gA aggregates (data not shown).

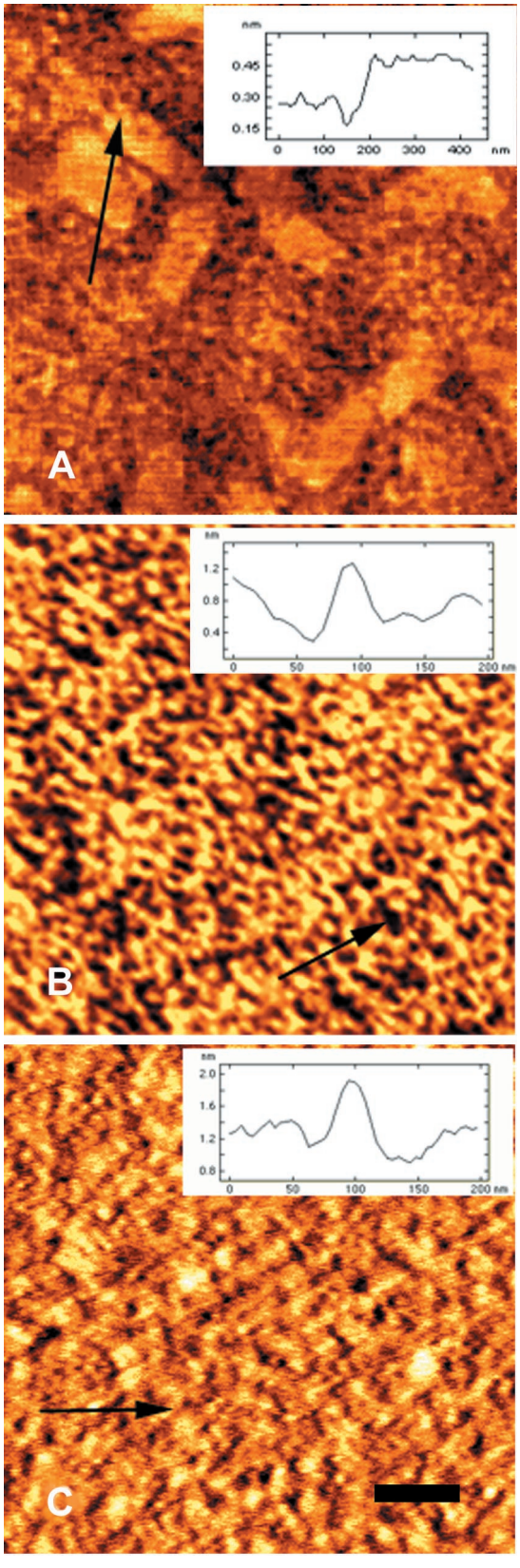

FIGURE 4 AFM images relative to gA-DPPC samples of gA concentration of (*A*) 4.4, (*B*) 5, and (*C*) 28 mol% (bar represents 200 nm). The arrows indicate the direction of line profiles reported in the insets.

In the sample at 8×10^{-2} mol%, it was also possible to identify small flat circular doughnut structures of $\sim 70 \pm 10$ nm in diameter, very similar in shape to the biggest one (Fig. 3 *B*). These small doughnuts were imaged as isolated units (Fig. 3 *E*) or aggregated (Fig. 3 *D*) to form bigger structures, very similar to doughnuts of 150-nm diameter. Spisni et al. (1983) suggested that gA aggregates in a lipid bilayer by hexameric gA aggregation. Mou et al. (1996) observed gA dimers of ~ 5 nm high spanning the lipid bilayer and arranged in hexameric base aggregation units of ~ 5 nm in diameter (Fig. 5 *B*).

On the basis of these considerations, a possible explanation of our observations for the more dilute samples (8×10^{-4}, 8×10^{-2} mol%) could be that gA aggregation in a DPPC monolayer is based on the proposed basic aggregation unit. As depicted in Fig. 5 *C*, six hexagonal units arranged in a circular ring can form a first doughnut of ~ 15 nm in diameter. This circular structure always has a hexagonal symmetry and can form, by a fractal growth, a similar circular ring of ~ 45 nm in diameter (Fig. 5 *D*). The same mechanism can lead to a 135-nm doughnut (not shown).

These theoretical diameters have to be convoluted with the so-called tip-locus-effect. In our experiment, we estimate an overall additive broadening of ~ 25 nm. The diameter of the basic aggregation unit becomes ~ 30 nm and the lateral dimension of the three theoretical doughnuts ~ 40, 70, and 150 nm, respectively. In conclusion, we think that the doughnuts appearing in our images are consistent with the presence of structures of 135 nm (Fig. 3, *A*, *B*, and *D*) and 45 nm (Fig. 3 *E*) in diameter.

Images for a sample of molar fraction of ~ 3.8 mol% (Fig. 3 *C*) revealed only small (70 nm) doughnut structures. This is probably because, near this concentration, the small doughnut structures begin to fill all available space, making it increasingly difficult to distinguish the bigger doughnut (i.e., structures of the subsequent order in fractal growth) dispersed in the DPPC matrix. The observation of gA aggregates reported in this paper may provide an explanation of the reduced mobility of gA monomers inserted in lipid bilayer, as measured by ionic conductance experiments (Mobashery, 1997).

Figure 4 shows images for samples of molar fraction of 4.4, 5, and 28 mol%. According to thermodynamic data, which suggest that gA is mixed with DPPC molecules, the doughnut rounded features totally disappeared. Figure 4 *A* shows the occurrence of patches of flat DPPC zones surrounded by a new phase: a network made of small circular units of ~ 30 nm. At gA concentration of 5 mol%, the new phase is prevalent and lipid patches disappear. This observation is consistent with the occurrence of a percolation transition, as described by Mou et al. (1996) and Killian and de Kruijff (1985) around 5 mol%. The new phase can be interpreted as a network made of basic gA aggregation units of ~ 5-nm diameter, broadened to ~ 30 nm (Fig. 5 *B*), mixed with DPPC molecules. The network seems to be more

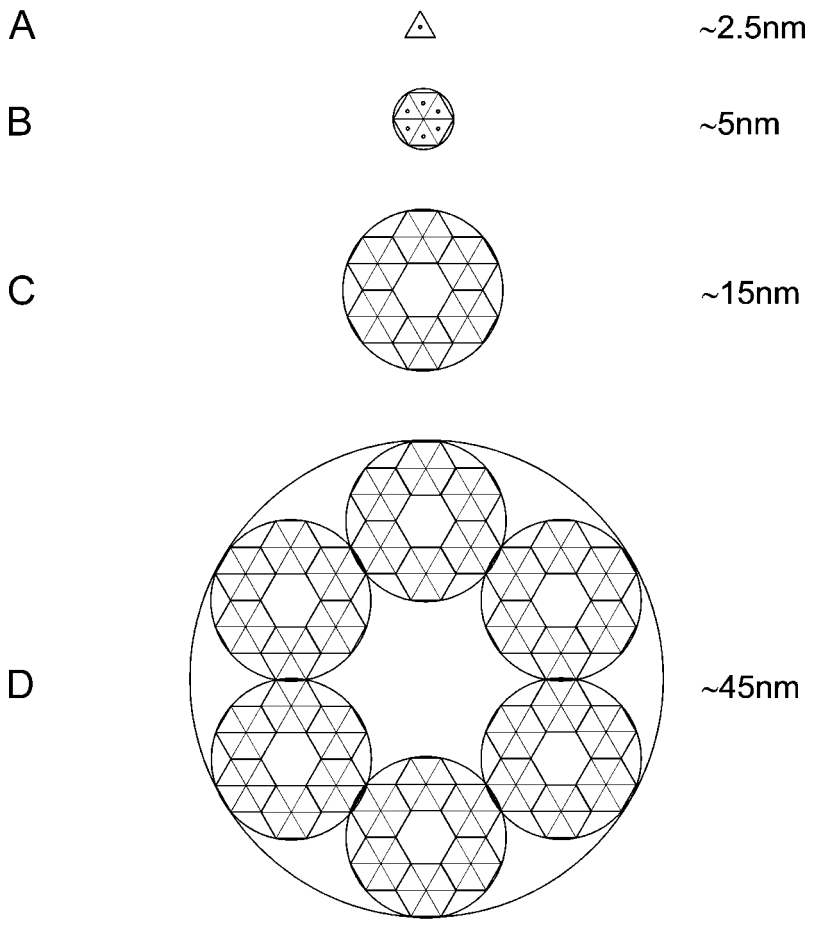

FIGURE 5 Horizontal growth model for gA in DPPC monolayer and theoretical diameters. (*A*) Single gA molecule. (*B*) Basic aggregation unit (gA hexamer). (*C*) Fractal aggregate of first level. (*D*) Fractal aggregate of second level. The broadening due to the tip-locus-effect leads to experimental diameters of ~30, 40, and 70 nm.

compact as the concentration increases from 5 to 28 mol% (Fig. 4, *B* and *C*, respectively).

Vertical growth

AFM images provide detailed information about the topography of the monolayers deposited at 35 mN m^{-1}. The height of the molecular structures were accurately measured by tracing line profiles (insets of Figs. 3 and 4). At very low gA concentration, line profiles show that gA aggregates rise from the lipid leaflet by ~0.5 nm from the top of DPPC molecules. These flat doughnuts dominate in the lowest gA concentration sample (8×10^{-4} mol%) (Fig. 3 *A*). When gA concentration increases, doughnut structures higher than 2 nm appear (inset of Fig. 3 *B*). These structures are abundant in the sample at a gA concentration of 8×10^{-2} mol% (Fig. 3 *B*).

To verify whether deposition pressure influences gA aggregate topology, the sample at 8×10^{-2} mol% was also deposited at 10 mN m^{-1}. The relative images (not shown) were similar to Fig. 3 *B*, except that they showed only flat doughnuts. We conclude that low deposition pressure prevents the formation of doughnuts higher than 0.5 nm. In general, it can be hypothesized that, when the fractal growth leads to the formation of doughnuts (up to ~3.8 mol%), the pressure at which the monolayer is deposited affects the heights of these gA aggregates. At 35 mN m^{-1}, a pressure generally accepted as a typical value for actual biomembranes and used in this work, flat (0.5 nm) and high (>2 nm) aggregates coexist.

A possible explanation of these findings could be provided by a simple model, depicted in Fig. 6, where SH hexamers (black box 2.5 nm in section in Fig. 6 and from above in Fig. 5 *B*) are bound to phospholipids according to the model proposed by Chiu et al. (1999) (where the H bonds between gA tryptophan and the DPPC glycerol group bind molecules to phospholipids). High structures could be due to vertical aggregates composed of two SH hexamers (5 nm high). The formation of these vertical aggregations can occur following the described HHSH dimer formation mechanism. This hypothesis is supported by the observation (from above) of the symmetry of the two models proposed in literature for the dimer formation, HHSH and DSDH. As reported by Spinsi et al. (1983) and more recently by Burkart et al. (1998), HHSH shows a triangular symmetry (Fig. 5 *A*). Conversely, observing the three-dimensional model proposed for DSDH (Burkart et al., 1998) from above, no symmetry appears. Because of the lack of trian-

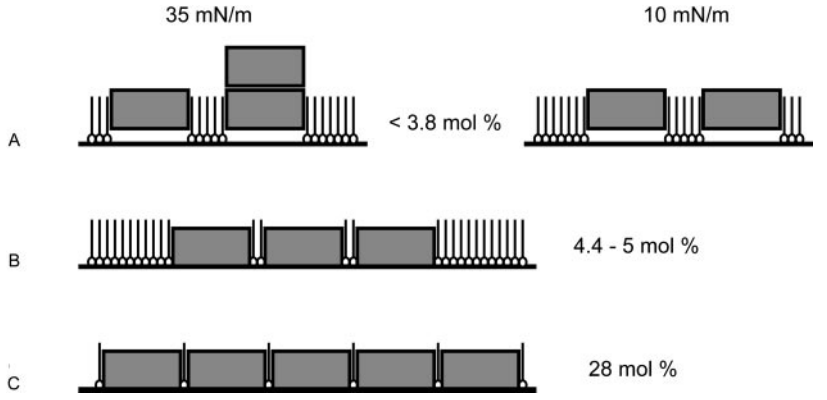

FIGURE 6 Vertical growth model for gA in DPPC monolayer. The pressure at which the monolayer was deposited is reported above the figure, whereas gA concentration is reported on the right. (*A*) Concentrations at which basic aggregation units (gA hexamers) aggregate following the fractal mechanism reported in Fig. 5. (*B*) Concentration at which a mixed gA-DPPC phase appears and a percolation occurs. (*C*) Maximum of miscibility between gA and DPPC.

gular symmetry, the aggregation of DSDH should not have hexagonal symmetry. Moreover, because of the compact structure of the DSDH, it is impossible to justify the height of only 2.5 nm that we measured for the lowest gA concentrations (corresponding to only one half of the total DSDH height). It is interesting to note that, after lowering the deposition pressure at 10 mN m^{-1}, only flat aggregates were observed. This demonstrates that the vertical growth is triggered by the deposition pressure and is based on the addition of SH to SH. In principle, we cannot rule out that high structures were SH aggregates on the top of the lipid monolayer, rather than dimeric structures embedded within the monolayer. However, it seems unlikely that the surface pressure can induce the rearrangement of two isolated SH in the DSDH structure.

Finally, after the disappearance of doughnuts, the mixed-phase gA-DPPC seems to be made of SH. In fact, it is possible to compare the heights of the mixed-phase and the DPPC monolayer. Line profiles (Fig. 4 *A*) demonstrate that the thickness of the mixed phase is lower than or equal to 3 nm (typical value for a DPPC monolayer).

CONCLUSION

Our experimental results indicate that, in contrast with previous observations (Killian and de Kruijff, 1985) gA aggregation does occur in DPPC monolayers even at very low molar fraction (8×10^{-4} mol%). The aggregation process seems to be mainly in the horizontal plane, based on SH hexameric units (Fig. 5 *B*) that aggregate following a growth fractal pattern, leading to the formation, on different scales, of typical doughnuts. Such gA aggregates could explain the reduced mobility of gA SH in a lipid bilayer, as proposed by Mobashery et al. (1997). The fractal mechanism leads to typical doughnuts up to 3.8 mol%. At 4.4 mol%, doughnuts totally disappear and a new phase appears. This is a mixed gA-DPPC phase that, at 5 mol%, is responsible for a percolation transition in agreement with the Mou et al. (1996) observation. This mixed phase is organized as a network of small hexameric gA aggregation units, constituted by SH.

Overall, our results are in good agreement with the aggregation model based on the hexameric base aggregation unit and with the percolation transition occurring at ~5 mol%, as previously proposed in literature. Moreover, for the first time, we reported on fractal grown pattern leading to peculiar doughnut features. With respect to the controversy concerning gA pore formation in a lipid environment, our data seem to rule out the occurrence of DSDH dimers.

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