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GLOBULIN 11S AND ITS MIXTURE WITH L-DPPC AT THE AIR/LIQUID INTERFACE

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Abstract

Langmuir films of globulin 11S protein, L-DPPC, and mixtures of both on water and on buffer subphases were studied. Brewster Angle Microscopy was used to characterize *in situ* the films morphology along π-A isotherms at the air/fluid interface. The L-DPPC monolayer on water behaved as has been reported extensively in the literature but a slight increase on surface pressure and a notable change in domain morphology is observed on buffer. This difference in domain behavior is due to the stabilization interaction of the LE phase by the buffer ions. On the other hand, the protein monolayer was prepared by direct deposit or injection below the surface. Both methods formed mostly a condensed film, with a multilayer formed by globular aggregates in the first method with the two subphases. However, the second method showed different behavior of the protein films depending on the subphase; on water the protein formed a homogeneous film with some globule aggregates, but on buffer a remarkably well organized monolayer was observed by AFM. Mixtures of globulin 11S and L-DPPC were prepared using both methods for the protein film formation at the air/fluid interface. BAM showed that the mixtures formed coexistence regions between two condensed phases, whose domains of both phases behave like liquids. Fingering phenomena was observed at the interface between protein-rich and L-DPPC-rich domains, which indicates that both phases are fluid. AFM images of the mixtures show indeed the formation of protein- or L-DPPC-rich domains. The liquid-like behavior could be explained due to different sizes of the protein and the L-DPPC, the minority compound in each kind of domain produces defects making them behave as liquids. Interestingly enough, as the monolayer is compressed to higher surface pressure, the lipid molecules are squeezed out and complete separation of the protein and L-DPPC is produced. Furthermore, we present evidence that the protein/L-DPPC mixtures produce films with holes, which might indicate its tendency to form hollow aggregates that could have some relevance in water-channel formation for *in vivo* seed germination.

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INTRODUCTION

Langmuir monolayers have been widely used as biological model membranes[1]. In particular, phospholipid monolayers are of great interest as model membranes, mainly due that phospholipids, as well as proteins, are the main structural elements of cell membranes. Surface and interfacial phenomena involving proteins are quite common and of great importance in nature and technology. Therefore, a detailed study of these phenomena is of great interest in biology, chemical technology, biotechnology, and offers new paths in the understanding of protein and polymer chemistry, environmental science. For example, there have been a wide range of studies about protein interface adsorption in a lipidic Langmuir monolayer, although these studies have used mainly food proteins, such as soy[2], sunflower[3], Ovoalbumin[4], β-lactoglobulin[5,6,7], etc. Indeed, it is possible to study the protein penetration in a lipidic monolayer as a function of superficial pressure; these provide some information about the interactions and structure of protein-lipid layer.

Cornell and Patterson[8] observed the penetration of β-lactoglobulin into mixed Langmuir monolayer of POPC and POPG, at a higher surface pressure than that shown by the pure protein solution, but only a small amount of protein was detected in the mixed films. They observed a binding of β-lactoglobulin at pH 4.4, which is when the protein carries net positive charge but not at higher pH. A similar observation was made by Bos and Nylander[9] for the interaction between β-lactoglobulin and DSPC and DSPA monolayers.

Protein adsorption at a clean air/water interface can also be studied directly as a function of surface pressure as a Langmuir film, which also provides information over the protein structure, interactions and conformation at a hydrophilic/hydrophobic interface. Some

examples of pure protein studies involve proteins such as bovine serum albumin[10], cytocrome c[11], apolipoproteins[12], etc.

Moreover, adsorption studies of proteins at fluid-fluid interfaces are essentially motivated by trying to elucidate structure functionality relationships in emulsifying and foaming food protein systems[13]. Hence, investigations of protein interfacial layers are of importance for understanding stability of emulsions and microemulsions when natural high molecular mass surface-active substances are applied as stabilizers. Colloid chemistry researchers have yet to get a better understanding on how proteins act as good stabilizers. For example, spatial structure of protein molecules governs their essential properties, including surface and biological activity, but in an emulsion or a microemulsion is not always clear if proteins are folded or unfolded.

Storage proteins in seeds are of prominent importance for supplying the world's protein requirements[14]. It has been shown that seed storage proteins are composed mainly by four fractions with different physicochemical properties. The major legume storage protein fractions are globulins, oligomeric globular proteins. The 11S globulins are composed of six non-covalently linked subunits, each of which contains a disulphide bridge pair of rather hydrophilic acidic 30-40 kDa α-polypeptide chain and another hydrophilic basis 20 kDa β-polypeptide chain. Thus, the molecular weight of the dimeric subunits is 50-60 kDa and therefore the hexameric protein 300-360 kDa. The oligomeric structure is stabilized by neutral salts in high ionic strength solution[15].

Amaranth (*Amarantus hypochondriacus*) is a cereal-like crop with high seed yield. The amaranth seeds has high proteins content (17.9 %)[15] with a better balance of essential amino acids than those found in cereals and legumes. Therefore, amaranth proteins are one of the more promising food ingredients, capable of complementing cereal or legume

proteins. Amaranth seeds have high proportion of globulins, 22 - 42 % and glutelins 14-18 % [16], the structure and functionality of these proteins present a strong dependency with the ionic strength, pH and temperature.

On the other hand, the most common way for studying Langmuir monolayers has been through the measurement of pressure–area isotherms, $\Pi(A,T) = \gamma^0(T) - \gamma(A,T)$, where T is the temperature, A is the area/molecule, γ and γ^0 are the surface tensions of the monolayer and of pure water, respectively. There are several techniques that can be also applied concomitant to observe the monolayer organization at the air/aqueous interface, such as polarized fluorescence microscopy (PFM)[17,18] and Brewster angle microscopy (BAM)[19]. These optical techniques are quite sensitive for observing very fine details in Langmuir monolayers, such as phase transitions, phase coexistence and molecular tilting. For example, these experimental techniques have revealed that singularities in the surface pressure–area isotherms are due to phase changes[20,21]. Both techniques have been widely used to observe the formation of domain structures on monolayers penetrated by proteins[2,4,7,22,23]. Their lateral resolution is, however, limited by the resolution of the optical microscope. The transfer of film from the air/aqueous interface to a solid support, using the well-known Langmuir-Blodgett (LB) film technique, can be used for further studies on the microstructure of the mixed protein-lipid film; The microstructure of the film in the solid support can then be imaged using electron microscopy[24,25] or atomic force microscopy (AFM)[26]. For example, the former technique has been used to study the miscibility of proteins and lipids at the air/water interface[27]. AFM has been used to study LB films (LB) of a widely range of systems such as DPPC[28], human proteins[29], and mixtures of DPPC with N-nitrosodiethylamine/bovine serum albumin[30]. Nevertheless, to the best of our

knowledge, neither microscope technique has been used for food protein monolayer penetration studies.

In this work, we have studied the formation of 11S amaranth globulin monolayers on water and buffer subphases and its interaction with L-DPPC in both subphases. In addition, the comparison of the results obtained when L-DPPC monolayers were deposited on both subphases is presented. The protein monolayers and its interaction with L-DPPC were done in two different ways: i) direct deposition on the interface and ii) injecting the proteins below the interface. Isotherm measurements and BAM observations along the compression isotherm were performed to follow the pure protein behavior and its interaction with L-DPPC. LB films were made to study the microstructure of the protein films and its mixture with L-DPPC using AFM.

EXPERIMENTAL SECTION

Protein extraction and purification, L-DPPC and subphases. *Amaranthus hypochondriacus* L. seeds, cv. Nutrisol were used for 11S globulin fraction isolation according to Barba de la Rosa et. al.[31]. Briefly, suspensions of defatted flour/extracting agents (1:10 w/v) were stirred for 1h at room temperature and centrifuged at 9000g for 20 min. In a sequential order; albumins were extracted with water, the precipitate was resuspended in 10 mM phosphate buffer, 1 mM EDTA, pH=7.5, containing 0.1 M NaCl. A second extraction was done with the same buffer and finally the pellet was wash with deionized water. All supernatants were mixed together and named as 7S globulins. The resulting precipitate was resuspended in 10 mM phosphate buffer, 1 mM EDTA pH 7.5 containing 0.8 M NaCl, a second extraction with the same buffer was done with the final

wash with water. The supernatant were mixed and named 11S globulins. Quantification of proteins was done using the protein Assay (BIO-RAD, Lab Hercules, CA USA).

L-DPPC (1,2-Dihexadecanoyl-sn-glycero-3-phosphocholine, ≥99 %) was purchased from Sigma-Aldrich Inc, USA, and used without any further purification. L-DPPC was dissolved using chloroform (Sigma-Aldrich, USA ≥99.99 %) to prepare the spreading solution at a concentration 0.353 mg/ml. Ultrapure water (Nanopure-UV, Barnstead/Thermoline, *18.3 MΩ-cm of resistivity*) was used throughout all preparations.

Langmuir monolayers, Langmuir-Blodgett films and BAM observations. All monolayer isotherms and transferred LB films were prepared on a computerized Nima LB trough (601-BAM, Nima Technology Ltd., England) using a Wilhelmy plate to measure the lateral surface pressure, $\Pi = \gamma^{o}-\gamma$. Temperature was kept constant at 25 ± 0.2 ℃ with the aid of a water circulator bath (NESLAB, RTE-211, USA). The compression rate was *15* cm^2/min. All experiments were carried out in a dust-free environment room cleaned with a laminar flow hood. BAM observations were made along the isotherm measurements with a BAM I-ELLI 2000 (Nanofilm Technologies GmbH, Germany) apparatus, with a spatial resolution of ca. 2 μm. The interface was illuminated at the water Brewster incidence angle (53.13°) for BAM observations and the analyzer was rotated for best contrast and to look for anisotropies of the film. The NIMA LB trough and BAM were placed together onto an optical table. The monolayers were transferred at a dipper speed of 1 mm/min in an upward stroke mode at a prefixed surface pressure. The mica was freshly cleaved just before the deposition. The AFM (see below) scanning was performed just after LB preparation.

Globulin 11S films. Two different methods were used to form globulin monolayers, in the first one, the protein was deposited with a microsyringe on a deionized water subphase or

buffer subphase, after 4 hrs the monolayer was compressed. In the second method the protein was injected below the air/water or air/buffer subphases, in this case we waited 6 hrs. before compression, to allow protein diffusion and trapping by the surface.

Monolayers of L-DPPC with globulin 11S. Two methods to incorporate globulin 11S into L-DPPC monolayer[12] were used. In the first one, the protein was deposited on the subphase surface, water subphase or buffer subphase, drop-by-drop with the aid of a microsyringe. After 15 min, 250 μL of L-DPPC was deposited onto the surface, by dropping it dissolved in the spreading solution. In the second method, L-DPPC was spread on the subphase with the aid of a Hamilton microsyringe. We waited at least 20 min for chloroform evaporation before monolayer compression, the barriers were closed a little and the protein injected below the subphase surface passing a syringe into the bulk from the outside part of the barriers, to disturb as a little as possible the L-DPPC monolayer, then the barriers were fully opened again. After approximately 6 hrs, to allow protein diffusion to the surface and to be able to obtain significant surface pressure for isotherm measurements, the compression started. All experiments were repeated three times obtaining good reproducibility within ± 0.1 mN/m.

AFM observations. Transferred monolayer of both pure protein and its mixture with L-DPPC were scanned with a NanoScope IIIa AFM (Digital Instruments, California, USA.) with a J-scanner that has a maximum scanning area of *100 x 100 μm*. We worked in tapping mode to obtain topographic and deflection images, using Si_3N_4 tips with a typical force constant of $0.3\ Nm^{-1}$. 125 μm-length tips with nominal resonance frequency of 300 kHz and spring constant of 40 N/m were used at room temperature in air. The oscillation frequency for scanning was set 0.1-3 kHz below resonance. Low scan rates typically

between *0.5-1 Hz* were used and the images were analyzed using the Digital Instruments software. AFM images were first obtained at low magnification and once a proper area was found, the scanning was changed to a higher resolution. Both height and deflection images were usually good enough. Therefore, the AFM images presented here are height images unless indicated otherwise.

Results and Discussion

L-DPPC monolayer. Fig. 1 shows typical isotherms of L-DPPC monolayers at 25°C, on deionized water and on sodium phosphate buffer subphases. The isotherms on the buffered subphase are shifted slightly to higher surface pressures with respect those on water. These monolayers present two phase transitions; Gas-Liquid Expanded (G-LE) and LE-Liquid Condensed (LC)[12]. The G-LE coexistence is not shown. L-DPPC phase transitions onto a deionized water subphase have been widely studied, and one of the most interesting features is the formations of LC chiral three-arm domains in coexistence with the LE phase. BAM images show the characteristic three armed domains observed in L-DPPC Langmuir monolayer (see Figs. 1a and b)[32]. The shape of these domains depends on line tension, dipolar interaction and chiral contributions[33]. When we used a sodium phosphate buffered water subphase, BAM images didn't show the characteristic chiral domains, instead images show anisotropic domains, often with straight edges (see Figs. 1d and e). Similar observations on the shifting of isotherms to higher surface pressures and changes on domains morphology in monolayers of L-DPPC in the presence of different ions in the subphase have been reported[34]. They find that the LC phase is essentially unaffected by its interactions with the ions in the subphase while the LE phase is not. They propose that

changes in domain morphology are probably due to an entropic stabilization of the LE phase. We believe that this stabilization must change the LC-LE line tension, thus preventing the formation of chiral patterns. In addition, we can argue that this stabilization will made the LE phase more robust, which shift the L-DPPC isotherm to higher surface pressures, as observed experimentally. The LE-LC transition was characterized by coalescence of the LC domains on further compression, to make a uniform film on both subphases (see Figs. 1c and f). L-DPPC monolayer on deionized water subphase collapse at $\Pi \sim 44.5$ mN/m and $A \sim 41$ Å^2/molecule, while in the phosphate buffer subphase the collapse occurs at $\Pi \sim 41.5$ mN/m and $A \sim 42$ Å^2/molecule.

Globulin 11S Langmuir films. As it is explained below, isotherms indicate that 11S protein from amaranth seed can be adsorbed at the air/water and air/buffer interfaces. Isotherms where the protein solution was directly deposited at the air/water interface, T = 25° C, show a high amount of molecules trapped on the interface and it is reflected with a rapid increase in surface pressure upon compression, see Fig. 2. Isotherms on both subphases are qualitatively different despite its closeness, especially at the lower surface pressures. The isotherm of globulin 11S on a water subphase shows a small shoulder, which in lipid monolayers, such as phospholipids, would be indicative of a phase transition. However, this is not the case for the protein films since BAM observations did not give us any indication of a phase transition. In the case of the isotherm of globulin 11S on the buffer subphase, it shows a continuous increase with some light slope changes, but again, BAM observations did not show any phase transition; isotherms show breaks that might indicate some phase transitions, BAM observations ruled out any transitions since no coexistence or phase changes were observed, this is only a homogeneous film was observed

along the isotherms up to collapse (see Fig. 2). Furthermore, isotherms taken at 3, 4 and 6 hrs after depositing the protein on the surface were quite reproducible. This might indicate that the amount of protein trapped by the interface is constant in the time frame of the experiments. However, we think that not all the protein deposited was trapped by the interface. In the case of the water subphase, since the protein is not water soluble, the amount that went into the bulk might have aggregated and precipitated. This was corroborated after finishing the experiments and cleaning the bottom surface of the trough, where a good amount of protein was found on it. This protein precipitation was not observed when the buffer subphase was used, therefore we believe that the protein film trapped at the interface did not change much, because either the protein that went into the buffer subphase diffused slowly towards the interface, not changing the protein surface concentration, or the monolayers acted as a Gibbs monolayer, reaching an equilibrium concentration at the interface within the time frame of the experiments.

Isotherms indicate that the monolayer of protein deposited on water suffered fracture collapse at $\Pi \sim 39$ mN/m, but BAM observations indicated that continuous collapse occurred at lower surface pressures. Fracture collapse of the isotherm of globulin deposited on buffer occurs at higher surface pressures, $\Pi \sim 44$ mN/m, but continuous collapse also occurred at lower surface pressures as indicated by BAM observations. In general, BAM observations (Figs. 2a, 2b, 2d, 2e), showed that during monolayer compression only a homogenous phase with small three-dimensional aggregates was observed. However, continuous collapse was present at pressures over $\Pi > 15$ mN/m, defined by the formation of a high number of large three-dimensional aggregates and structures, see Figs. 2c and 2f, where the white structures are much thicker that the rest of the film. This collapse behavior

already had been observed in globulin 11S of soy seed films, under similar experimental conditions[35].

Fig. 3 shows the results when the amaranth 11S globulins were injected under the two working interfaces. Compression started six hours after injecting the sample. Isotherms and BAM observations in deionized water subphase showed that at low surface pressures a phase coexistence region between two fluid phases is present; the dark area would correspond to a Gas (G) phase and the white structures (2-D soap-froth and rounded domain structures) would correspond to a Liquid Expanded (LE) like phase (see Fig. 3a and b). The surface pressure was quite low during most of the compression, which can be attributed to the presence of the two-dimensional (2D) G phase. In this case a 2D G phase means a phase with low density of protein molecules (or regions of almost bare water). The protein was injected in the bulk a few millimeters from the surface and then a small amount of protein diffused towards the surface. Since globulins are not water soluble, probably most of the protein aggregated and precipitated to the bottom of the trough, for that reason the lateral pressure does not increase much upon compression. After removing the subphase, indeed precipitated protein was found on the bottom of the trough, as a protein film. BAM images show the existence of rings and soap-froths (see Figs. 3a and b) similar to those observed in colloidal systems[36]. At higher surface pressures, BAM images showed that the amaranth globulin 11S film was in a homogeneous condensed phase with few small 3D aggregates (Fig. 3c). It is worth noticing that in this case, the amount of protein trapped at the air/water interface was lower than the amount of protein trapped at air/water interface when the globulin was directly spread on the surface.

On the other hand, if the protein is injected under the air/buffer interface, the isotherms are very similar to those obtained when the protein is spread directly on the buffer subphase

(see Figs. 2 and 3). Therefore, the lateral pressure obtained in the protein sample injected in buffer is greater than that obtained if the protein was injected in a deionized water subphase, as it can be observed in Fig. 3. This indicates that a greater amount of protein diffuses towards the buffer surface and gets trapped. This different behavior might be due to the high solubility of the globulin in the phosphate buffer, which allows the transport of the protein in a more effective way, without aggregation and further precipitation. BAM observations showed that there weren't changes along the isotherm; this is, there is always a homogeneous and continuous protein monolayer, with a scarce number of 3-D aggregates (see Figure 3c, 3d y 3e).

Mixed films of Globulin 11S and L-DPPC

Fig. 4 shows the experiments where L-DPPC was spread on amaranth globulin 11S film at the air/water and air/buffer subphases. Π-A isotherms for the mixed films in both cases show a rapid increase in surface pressure upon compression, indicating that there must be a continuous mixed film at the interface; this is, only condensed phases are present. The protein presence causes the disappearance of the coexistence region of the characteristic LE-LC phases in L-DPPC isotherm monolayers. In this case, we performed experiments with waiting times before compression of 4, 6 and 8 hrs after deposition of the L-DPPC and isotherms were essentially reproducible within ± 1 mN/m.

Fig. 4a shows a BAM image at low pressures, c. a. Π ~ 0 mN/m, of the film on water subphase. On the top left corner of the image, one can observe a large domain that shows a bright fingering instability. The rest of Fig. 4a shows the formation of some small rounded domains, often connected, which show typical internal anisotropy due to molecular tilting.

Since globulin protein molecules are rounded when folded or disorganized when unfolded, domains with internal anisotropic structure must be formed mostly by L-DPPC molecules. Therefore, we proposed that upon compression, L-DPPC start being expelled from the mixed film like that located on top left corner of the image. We recognized this waving instability as Mullins-Sekerka instability due to the expelling of the L-DPPC molecules from the mixed film. The Mullins-Sekerka instability is associated with the mass transport throughout interfaces due to concentration gradients in mixtures, i.e. with the expulsion of impurities of a phase rich in one of the components, in this case the L-DPPC small molecules being expelled from the protein rich phase. In addition, we ruled out the presence of a G phase because the isotherm increases its surface pressure rapidly after compression. Therefore, the rounded domains, with internal anisotropy, must correspond to the L-DPPC LC phase while the continuous darker phase surrounding the small domains must correspond to the L-DPPC LE phase.

Fig. 4b shows regions with mostly L-DPPC phases, where the LC domains start to coalesce and the area covered by the LE phase becomes smaller. Remarkably, separation between protein rich and LC L-DPPC rich regions is completed at higher surface pressures. Fig. 4c shows a typical image of these large regions; since the protein molecules are bulkier and thicker than the phospholipid molecules, we assign the brighter regions as protein-rich regions and the dark regions as L-DPPC LC-rich regions. The large protein-rich regions show remnants of the Mullins-Sekerka instability along its interface with the L-DPPC rich region.

On the other hand, when L-DPPC was spread on the protein film supported on the buffer subphase, we observed the formation of a mostly continuous film at low surface pressure (see Fig. 4d). The film formed is a rather a good mixture of both amaranth globulin 11S

protein and L-DPPC. However, at intermediate surface pressures, c.a. 15 mN/m, we observed again the separation of protein- and L-DPPC-rich regions as shown in Fig. 4e, but in this case no Mullins-Sekerka instability was observed. The thicker large gray island on the top of this image corresponds to the protein-rich region while the dark region must be the L-DPPC LC-rich region. The protein-rich island contains imbedded some large white areas that are elongated in the same direction. Not capture by the still images, is the relative movement of the protein-rich island and the L-DPPC region. Since the amaranth globulin 11S is more stable in buffer than in water, i. e. it retains its three dimensional globular shape, our observations lead us to propose that the protein-rich island acts like an ultrathin granular film rather than a condensed compact region; this is, the island is like a two-dimensional granular fluid. Thus the island relative movement produces shearing, elongating the white domains within it in the shearing direction. The granular nature of domains was corroborated by AFM observations, as discussed below. Furthermore, Fig. 4f shows clearly domains of protein-rich (whitest domain), mixed domains (granular grey region) and L-DPPC-rich region (dark region). Again, no instability was observed at the border of the protein-rich and L-DPPC-rich interface. However, the mixed region shows patterns that resemble spinodal decomposition or a microphase separation, indicating the separation of the protein and lipid molecules. The lack of an instability at the border of the protein-rich regions in the experiments on a buffer subphase, indicates that the separation mechanism differs from that on the water subphase. It is possible that the differences arise because the protein on the buffer subphase acts more like a granular material while on the water subphase might be acting like a melt due to its tendency to unfold in water.

Fig. 5 shows isotherms and BAM images when a globulin 11S solution was injected beneath the preformed L-DPPC monolayer at deionized water and buffer surfaces.

Compression started ~ 4 hrs after the protein was injected. The surface pressure in both isotherms started to rise as soon as the systems were compressed, and no flat LC-LE coexistence region of L-DPPC (see Fig. 1) was observed. Both isotherms are very similar up to about 4 mN/m and 85 $Å^2$/molecule, where the isotherm obtained on the water subphase becomes more compressible up to about 10 mN/m and 60 $Å^2$/molecule, after which the situation reverses and the film becomes less compressible than the one obtained on the buffer surface. Finally, the pressure readings of the isotherm obtained on the water surface surpass those of the isotherm obtained on the buffer surface at about 17 mN/m and 50 $Å^2$/molecule. In both subphases, BAM images show the presence of small domains of the protein/L-DPPC mixture at low surface pressures, as shown in Figs. 5a and d. The L-DPPC LC domains on the water subphase are somewhat bigger, and with small instabilities at the borders, than those observed on the buffer subphase that show a more stable straight borders, similar to those observed without protein (see Figs. 1d-e). In addition, domains show internal tilting anisotropy, which indicates that they are made mostly by L-DPPC molecules and correspond to the LC phase. Thus the continuous dark areas must also correspond to the phospholipid LE phase.

At low surface pressures, BAM images gave little evidence of the presence of the globulin 11S on the L-DPPC monolayer. However, with further compression, c. a. $\Pi > 15$mN/m, the LC domains grow further at the expense of the LE phase and some white dots become evident. We think that these white dots are evidence of the protein presence. Furthermore, the instabilities in the LC domains on the water surface become larger and LC domains observed at the buffer subphase start to developed small instabilities (clearly observed at high magnification). We proposed again that they are Mullins-Sekerka instabilities, but in these cases the protein molecules are being expelled from the LC phospholipid domains.

The instabilities are larger in the LC domains at the water surface because they contain a higher concentration of protein than the LC domains obtained in buffer, since ions stabilize the LE phase, as discussed above, making difficult the protein penetration/interaction with the LE phase of the monolayer in the latter case.

At higher surface pressure, we found evidence of protein monolayer inside of the LC domains as well as at the LC-LE interface in the experiments on the water subphase. It is strange that almost no evidence of protein is observed in the LE phase. It is possible that in this case the protein is unfolded, making it difficult to distinguish from the L-DPPC LE phase. However, the protein is almost entirely located at the LC-LE interface in the experiments done on buffer. In general, we believe that more protein penetrates/interacts with the L-DPPC monolayer on water than on buffer. These facts and assumptions would explain the different behavior of the isotherms of Fig. 5; at the beginning the monolayer on buffer is slightly more rigid than that on water, due to buffer ion stabilization of the LE phase, making it less compressible. This stabilization also prevents the penetration/interaction with the globulin having more protein molecules in the experiments on water than in the experiments on buffer. Therefore, the compressibility changes midway the compression process, because on water we will have more total number of molecules at the interface than on buffer.

AFM Results.

Langmuir-Blodgett films were prepared for each one of the different experiments for further analysis by AFM. For completeness of the present work, we also present the case when pure L-DPPC was deposited on a deionized water or a buffer subphase, the transfer process was done at a lateral pressure of Π=14 mN/m. This pressure was chosen above the

LC-LE transition, as BAM images show that the L-DPPC Langmuir monolayer on both subphases is in the pure LC phase. At this pressure, the characteristic LC domains have coalesced, forming a uniform phase, with only defect lines and small holes remnant of the LE phase, as shown in Fig. 6: These holes and lines could not be observed by BAM because they are beyond its resolution. Fig. 6a show a flat film with a high of ≈ 2.8 nm with respect to the mica surface, but also reveal the presence of curved lines of smaller height, z ≈ 2.3 nm; these lines could correspond to the union (healing lines) between two or more chiral LC domains. L-DPPC molecules have a size of ≈ 3nm, therefore we can assume that the thicker flat and continuous phase correspond to LC phase while the darker lines correspond to a metastable remnant of the LE phase. AFM images for the monolayer spread on a buffer subphase (see Fig. 6b) show that the healing lines are not curvy as those of Fig. 6a. This is in good agreement with BAM observations which show that on a water buffer the LC domains are chiral, while those on the buffer subphase are not. We observed an unusually large relative difference in height between the LE and LC phase of the film of about 1 nm, while on water this height difference is about 0.5 nm. This LC-LE height difference might be explained as follows: The LC phase of L-DPPC on buffer does not produce chiral structures, therefore it might have a lower tilt angle than the LC phase that produces chiral structures on deionized water. In addition, the buffer ions might only penetrate the LE phase but not the LC phase[34], since the phosphate ions are rather large, the L-DPPC molecules will be more separated from each other in the LE phase on buffer than on water, giving the aliphatic chains more room to sample more configurations, thus making the LE phase thinner on average, increasing the height difference between the LC to LE phases on buffer with respect to the same phases on water.

Fig. 7 presents the images of globulin 11S transferred from both water and buffer at Π ~ 18 mN/m. Two aspects are immediately observed, one is that the films is not a monolayer, since many aggregates are formed, and the second is that those aggregates are globular. However, the globules formed on water are somewhat bigger than those globules formed on buffer, clearly confirming the tendency of globulin 11S to aggregate in water, while the phosphate buffer tends to stabilize the individual proteins. Most of the protein aggregates on water lie in the size range between 50 to 70 nm (see Figs. 7a-b). Figs. 7c-d show images of globulin 11S deposited on buffer. It was noticed that they form smaller globular structures than the sample deposited on water. The sizes of these globules range between 25 to 50 nm. The sizes of the smaller globules might correspond to the actual protein size, thus it is clear from the images that the buffer does really gets the protein dispersed and avoid its aggregation. In general, globular structures were expected given the rounded nature of globulin 11S.

AFM images when globulin 11S was injected below the interface and transferred at Π ~ 8 mN/m are presented in Fig. 8. Note that in the film transferred from the air/water interface the globular structures are scarce, this is a smooth film was observed as shown in Figs. 8a and b instead of the globular structures shown in Fig. 7. It is possible that the protein is unfolded in a deionized water subphase, therefore when it is trapped by the interface proteins formed a two-dimensional fluid- or melt-like film. On the other hand, films extracted when the globulin was injected below the air/buffer interface and allowed to diffuse to the interface, showed mostly a dense monolayer formed by globular protein structures, see Figs. 8c and d, i.e. the protein is not unfolded. Interestingly enough, the film is highly ordered, with crystal-like two-dimensional order: the inset in Fig. 8d is the Fourier

transform of the image, which qualitatively shows a good order of the protein monolayer. The monolayer contains defects in the form of small three-dimensional aggregates (white dots) and vacancies, however. The present of these defects might explain the high compressibility of the monolayer observed in the isotherm, see Fig. 3. Nevertheless, our results show that one can build well ordered, two-dimensional crystal-like structures out of globular proteins, similar to those formed with metallic nanoparticles[37], which can have potential applications in technology, such as molecular electronics.

Fig. 9 show images obtained when globulin 11S was deposited on air/water or air/buffer interfaces and immediately a L-DPPC solution was spread on it. When the protein was deposited on a deionized water subphase, Figs. 9a)-c), it is clearly observed the formation of coexistence regions; protein- and L-DPPC-rich regions; the small-rounded domains and the large domain at the bottom of the image correspond to protein-rich regions, where one can easily observe that protein globules are the main structures of these domains. The rounded domains are surrounded by a continuous, shallower phase that contains a good number of globular protein structures, but from the height of the majority phase we identify it as composed mainly of L-DPPC molecules. Fig. 9c) is a close up image of one of the protein-rich domains, typically these domains shows wiggles at their borders, reminiscent of an instability, probably due to the expelling of L-DPPC molecules from their interior upon compression, in agreement with BAM observations (see above). In addition, the protein-rich phase shows the formation of holes that are discussed below. Figs 9d)-f) shows the mixture on buffer subphase. The AFM images show a fracture film; apparently L-DPPC forms fractal-like channels in the protein-DPPC mixture that percolates the image. In addition, in this percolated phase one can distinguish globules embedded in a continuous lipid film and the formation of three dimensional aggregates. We believe that these fractal-

like channels help the DPPC molecules to move out of the protein-rich domains during compression, thus avoiding the formation of Mullins-Sekerka instabilities at the protein- and DPPC-rich domains interface. Furthermore, these channels will also make these protein-rich domains labile against stress forces, making act as a two-dimensional liquid or granular film, in agreement with BAM observations discussed above.

In addition, large protein-rich domains like the one at the bottom of Figs. 9a) and b), show a remarkable internal nanohole structure: Fig. 10 shows a typical higher magnification obtained from these large protein-rich regions, in which we can observe the presence of aggregates with a hole at the center. These aggregates have an average size of 22.38 ± 1.55 nm and the average size of holes is 8.1 ± 1.19 nm. It is believe that this kind of proteins form channels *in vivo* for water transport during seed germination, and therefore, the presence of holes in mixtures of this protein with DPPC might evidence its tendency for channel formation.

Finally, we show the images obtained by AFM of the experiments in which the protein was injected below a L-.DPPC monolayer; the monolayer was deposited on a deionized water or on buffer subphases, see Fig. 11. The images 11a-c correspond to the case of a water sub-phase, for which we waited 6 hr before compression to a given surface pressure for LB transfer; in order for the globulin to penetrate or interact with the L-DPPC film. In images 11a-b, we observed the presence of small aggregates that form short fibril structures. On higher magnification of the image, we observed that the aggregates that form fibers were united by a continuous shallower phase than the aggregates, possibly unfolded protein, which can be clearly observed in image 11c. In this case, due to the properties of the

globulins in water, the continuous phase could be a mixture of unfolded protein and DPPC, but no clear evidence of DPPC-rich domains was found.

A very different behavior was observed when a buffer subphase was used; no fiber formation was observed. In addition, large domains that appear quite flat corresponding to the LC phase of L-DPPC were observed, although the height average of these domains is of approximately 6 nm, which is approximately twice those of L-DPPC monolayer, indicating that they correspond to a bilayer. Therefore, this bilayer must onto top a monolayer, with the hydrophobic tails of the top layer directed towards the air. Furthermore, the LC domains of the bilayer are irregular and much less protein globules are evident even in the monolayer regions. This behavior, at the transfer pressure of 18 mN/m, is in agreement with isotherm and BAM observations, which show limited presence of proteins with the L-DPPC monolayer.

CONCLUSIONS

In this work, we show that pure globulin 11S proteins form films on the surface of deionized water and of buffer subphases. Two different methods were used to test protein film formation: the first one was direct depositing of the protein on the surface and the second was by injecting it on the subphase below the surface. The first method produced globular aggregates of the protein, although the globules formed in buffer were mostly of molecular size. The second method, however, produced very different results: isotherms in deionized water show a much less amount of globular structures than in buffer. In addition, AFM images showed that in water the protein might have unfolded, forming a smooth film, while in buffer the protein formed a remarkably, well organized, crystalline-like two-dimensional film. This later organization must be due to the globular nature of the protein,

which in buffer behaves as a nanoparticle. In fact, such type of organization has been shown in colloidal systems[36] and metal nanoparticles[37] at the air/water interface. Therefore, our procedure can be used to prepared well organized or even crystalline-like films of globular proteins, which can be used for protein crystal structure analysis or for technological application based on well-organized protein arrays.

On the other hand, when an L-DPPC film is deposited on a globulin film, the isotherm behavior is similar on both subphases, but at higher surface pressure the L-DDPC is squeeze out forming protein- and L-DPPC-rich domains; while L-DPPC is squeeze out, Mullins-Sekerka instabilities are formed at the edge of the domains in experiments on water. Therefore, the experiments indicate that protein-lipid mixture should not be used at relatively high pressure, because they might separate. Furthermore, AFM observations revealed that the protein-rich domains formed nanoholes, which might be taken as an indication of the natural tendency of the protein mixture with lipids to form such structures, which might also have some implications for water transport *in vivo* during seed germination.

When the L-DPPC Langmuir monolayer is first formed and the protein is injected in the subphase, BAM observation revealed little penetration of the protein, even in the LE phase. At higher compressions, the protein seems to accumulate at the LE-LC interface in both subphases. AFM images confirmed these observations, but it seems that there is more protein penetration in water that in buffer. This difference can be understood because of the increased stability of the L-DPPC Langmuir monolayer by the buffer ions, decreasing the protein penetration. Furthermore, the protein-L-DPPC mixture seems to develop small fiber-like structures on water; fiber formation is very important in the food industry, and

our experiments suggests that amaranth 11S globulin could make textures similar to globulin 11S from soybean[38].

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FIGURE CAPTIONS

Fig. 1. (Left panel) Π vs A isotherms of L-DPPC monolayers spread on a phosphate buffered water subphase (pH 8.0 and 25°C, solid symbols) and on a deionized water subphase (pH~5.5 and 25°C, open symbols). (Right panel) BAM images corresponding to the arrow positions in the left panel. a) Chiral LC domains on a continuous LE phase on a water subphase; b) The number and size of the three armed chiral domains of the LC phase increase. c) Pure LC phase. d) LC domains on a continuous LE phase observed on a phosphate buffered water subphase. Note that the LC domains do not longer have the chiral shape but they have sharp edges, which are more noticeable in e). f) The LC domains are coalescing to form a continuous LC single phase. Each image size is 462 μm x 564 μm.

Fig. 2. (Left panel) Π vs A isotherms of globulin 11S film spread onto a deionized water subphase at T=25°C (solid symbols). Globulin 11S spread onto a phosphate buffered water subphase at T= 25°C (open symbols). (Right panel) BAM images a) y b) Globulin 11S spread on deionized water subphase showing a homogeneous phase with small three-dimensional aggregates; c) Collapse of the globulin 11S monolayer on water subphase. d), e) and f) Globulin 11S monolayer deposited on a phosphate buffered water subphase, bright points and foam-like structure must correspond to three dimensional protein structures. Each image size is 462 μm x 564 μm.

Fig. 3. (Left panel) Π vs A isotherms of globulin 11S monolayers obtained 6 hrs after protein injection below the air/buffer (pH 8.0 and 25°C, solid symbols) and the air/water (pH ~ 5.5 and 25°C, open symbols) interfaces. (Right panel) BAM images corresponding to the arrow positions in the left panel. a)-b) Coexistence between the LE-G phases on the water subphase; the small circular domains correspond to the LE phase and the dark, continuous area is the G phase. c) Continuous LE phase with small three-dimensional aggregates. d)-f) An almost homogeneous and continuous protein film is formed at all surface pressures, when the protein is injected below the air/buffer interface and allow to diffuse towards it. Each image size is 462 μm x 564 μm.

Fig. 4.- (Left panel) Π vs A isotherms of L-DPPC monolayer spread on a globulin film at the air/water interface (T= 25 °C, open symbols) and on a globulin film at the air/buffer interface (T= 25°C, solid symbols). (Right panel) BAM images corresponding to the arrow positions in the left panel. a) Coexistence between a L-DPPC-rich region and mixture region. L-DPPC domains do not show chiral features due to protein presence. Note that the domain on the top left corner present a fingering instability; b) coalescence of L-DPPC domains, the continuum formed by these domains present internal anisotropic structure due to molecular tilt of the L-DPPC molecules; c) Clear separation into a protein-rich (white) region showing fingering and L-DPPC-rich (dark) region. d) At low surface pressures L-DPPC and Globulin mix well; e) Coexistence between a protein rich-domain, brighter top domain, and a L-DPPC-rich phase, bottom dark region; f) image clearly showing a protein-rich domain (white), a L-DPPC-rich region (dark) and a mix domain of both protein and L-DPPC (gray domain). Each image size is 462 μm x 564 μm.

Fig. 5. (Left panel) Π vs A isotherms of a Langmuir monolayer of L-DPPC when globulin11S was injected below it. L-DPPC was spread on the water subphase (T= 25 °C, open symbols) and on the buffer subphase (T= 25°C, solid symbols). (Right panel) BAM images corresponding to the arrow positions in the left panel. a)-c) LC domains of L-DPPC monolayer spread on water; the domains show fingering instabilities. In

addition, one can observe small white dots corresponding to protein aggregates; in c), the protein concentrates mainly in the LE-LC interface of the L-DPPC monolayer. d)-f) L-DPPC domains on buffer subphase; the LC and LE phases of L-DPPC show small white dots imbedded corresponding to the protein; in f), the protein aggregates at the LE-LC interface, but the protein aggregates are smaller that in experiments done on water subphase. Each image size is 462 μm x 564 μm.

Fig. 6. AFM images of L-DPPC transferred onto mica at Π = 14 mN/m. a) transferred from a deionized water subphase. The continuous phase corresponds to the LC phase while the phase made of small, darker circles and lines corresponds to a metastable LE phase. The height of each phase with respect to the mica is ~2.8 nm and ~ 2.3 nm for the LC and LE phases, respectively. Note that the curved lines come from the healing process among the chiral LC domains. b). transferred monolayer from the buffer subphase. In this case the healing defect lines are not so curved as in a). Moreover, the height difference between the phases is bigger; the height of the LC, continuous phase, is ~2.6 nm, while the darker, metastable LE phase, is ~1.6 nm.

Fig. 7. AFM images of globulin 11S transferred onto mica when the protein was spread on deionized water (a-b) and buffer subphases (c-d). The transfer pressure was Π = 18 mN/m. a) Figure shows bright dots that correspond to globular structures of different sizes; b) is a zoom were one can clearly observe these globular structures forming a multilayer. c) Transferred film from a buffered water subphase. In this case, it was observed similar structures than those observed on water subphase, but the globular structures have smaller dimensions; d) Globular structures mostly formed by individual proteins.

Fig. 8. AFM images of globulin 11S transferred onto mica when the protein was injected below the interface and allowed to diffuse for six-hours towards the interface. The transfer was done at Π = 8 mN/m a) and b) air/water interface; c) and d) air/buffer interface. On both subphases a homogeneous film is formed; on buffer the film shows a remarkable crystalline-like order. The inset in d) is the Fourier transform of the figure.

Fig. 9. AFM images of a mixture of globulin 11S and L-DPPC transferred onto mica when: a)-c) globulin was deposited on deionized water subphase and d)-f) globulin was deposited on a buffer subphase; L-DPPC was immediately deposited in both cases. The transference pressure was Π = 18 mN/m. a) Coexistence between protein-rich domains and L-DPPC-rich region; b) Close-up of image a) where one can see that the large domain at the bottom presents internal structure, i. e. holes (see also Fig. 10); c) Protein rich domains show globular internal structure; d)–f) the mixture shows a fracture film; apparently L-DPPC connects the protein globules. In addition, three dimensional aggregates are common.

Fig. 10. AFM images of a mixture of globulin 11S and L-DPPC transferred on mica. Globulin was deposited on deionized water subphase and immediately L-DPPC was spread. Transference was done at a pressure Π = 18 mN/m. Image shows aggregates with a hole at the center, these aggregates have an average size of 22.38 ± 1.55 nm, and the average size of the holes is 8.1 ± 1.19 nm.

Fig. 11. Typical AFM images of a mixture of globulin 11S and L-DPPC transferred onto mica when: a)-c) globulin was injected below the water subphase and d)-f) globulin was injected below the buffer subphase; L-DPPC was deposited before injection of the protein in both cases. The transference pressure was Π = 18 mN/m. a)-c) a granular mixture is formed: the protein shows a globular structure and penetrates the L-DPPC monolayer. d)-f) the LC domains of L-DPPC are irregular and much less protein globules are evident.

FIGURES

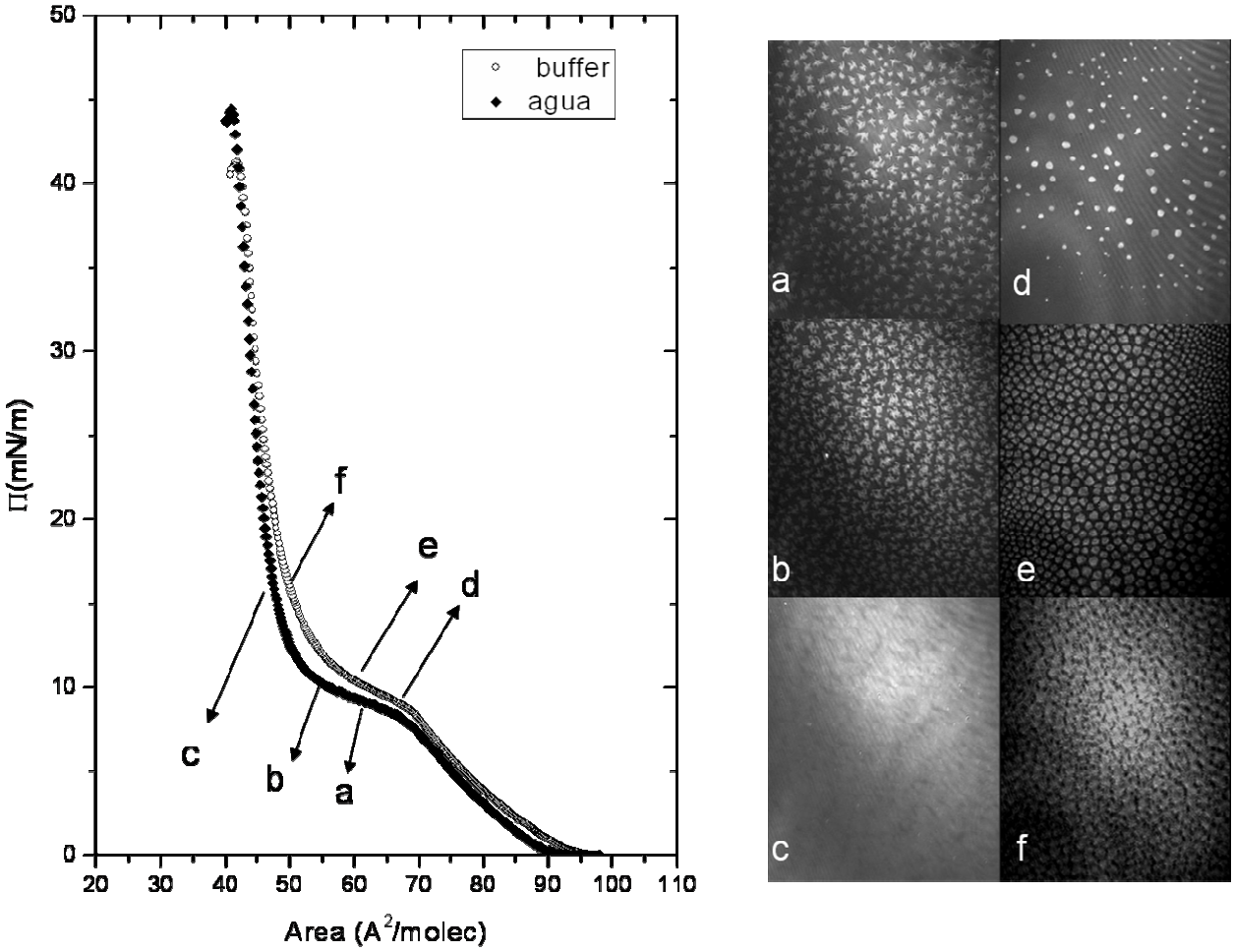


Figure 1 Garcia-Gonzalez *et. al.*

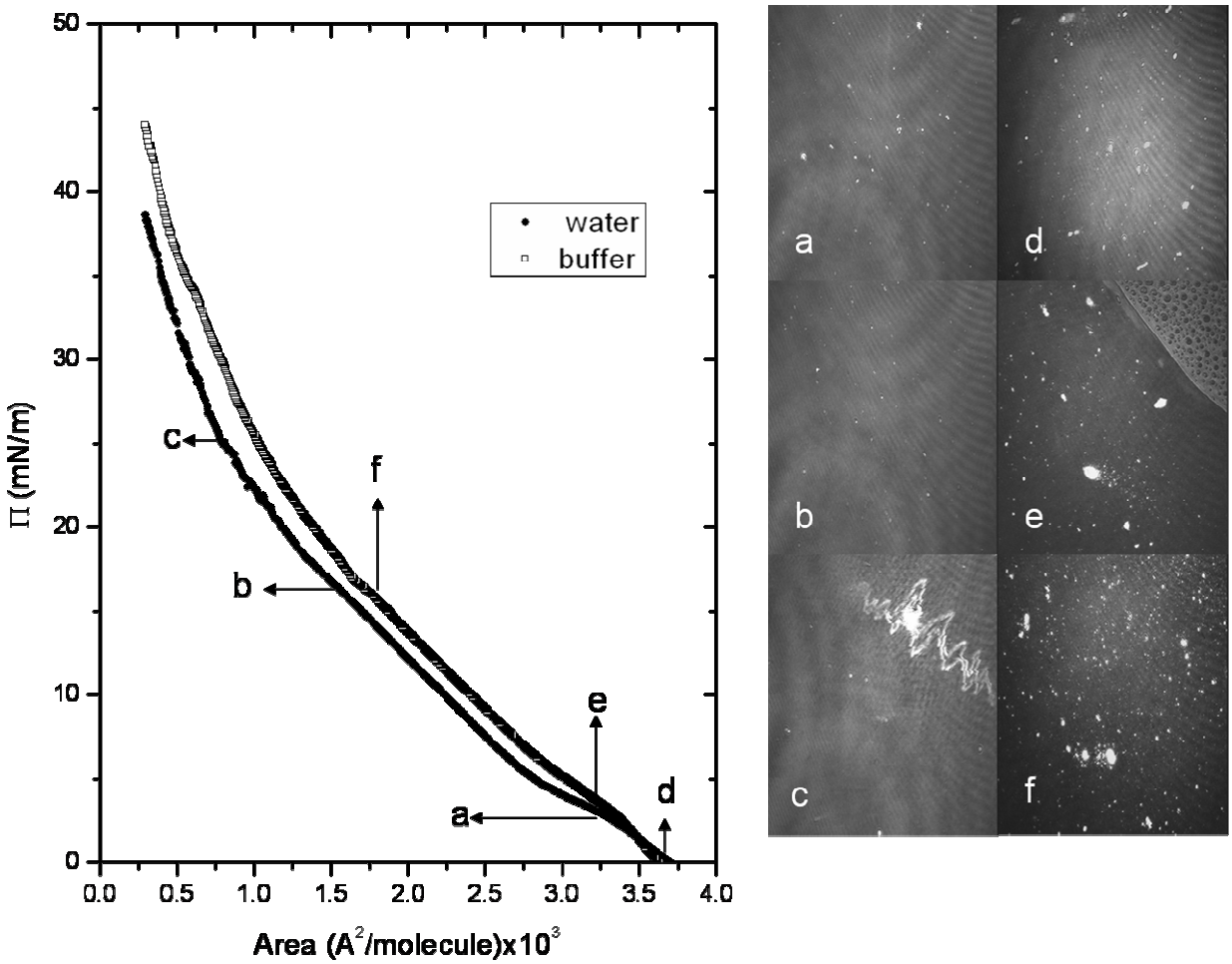
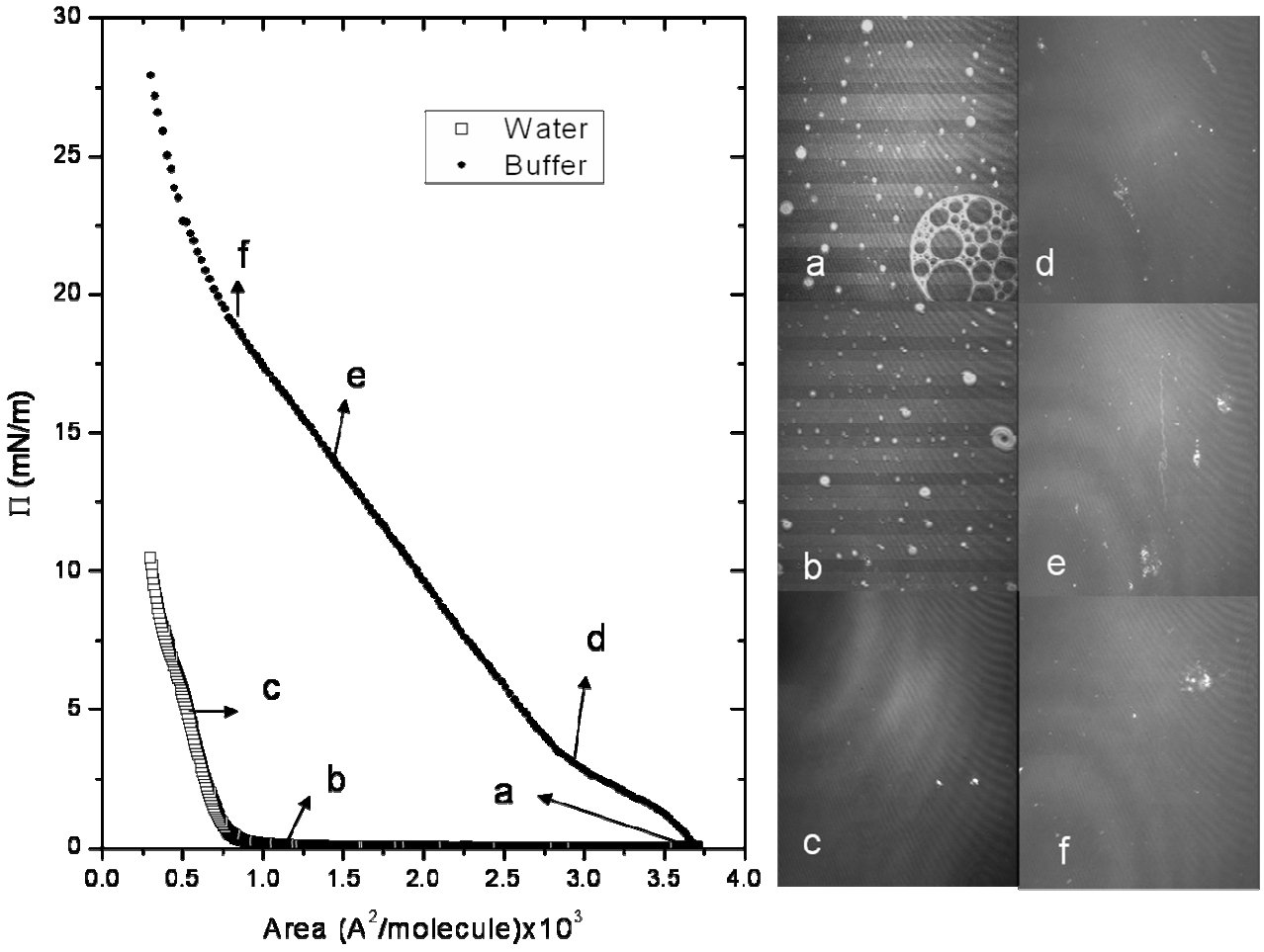


Figure 2. Garcia-Gonzalez *et. al.*



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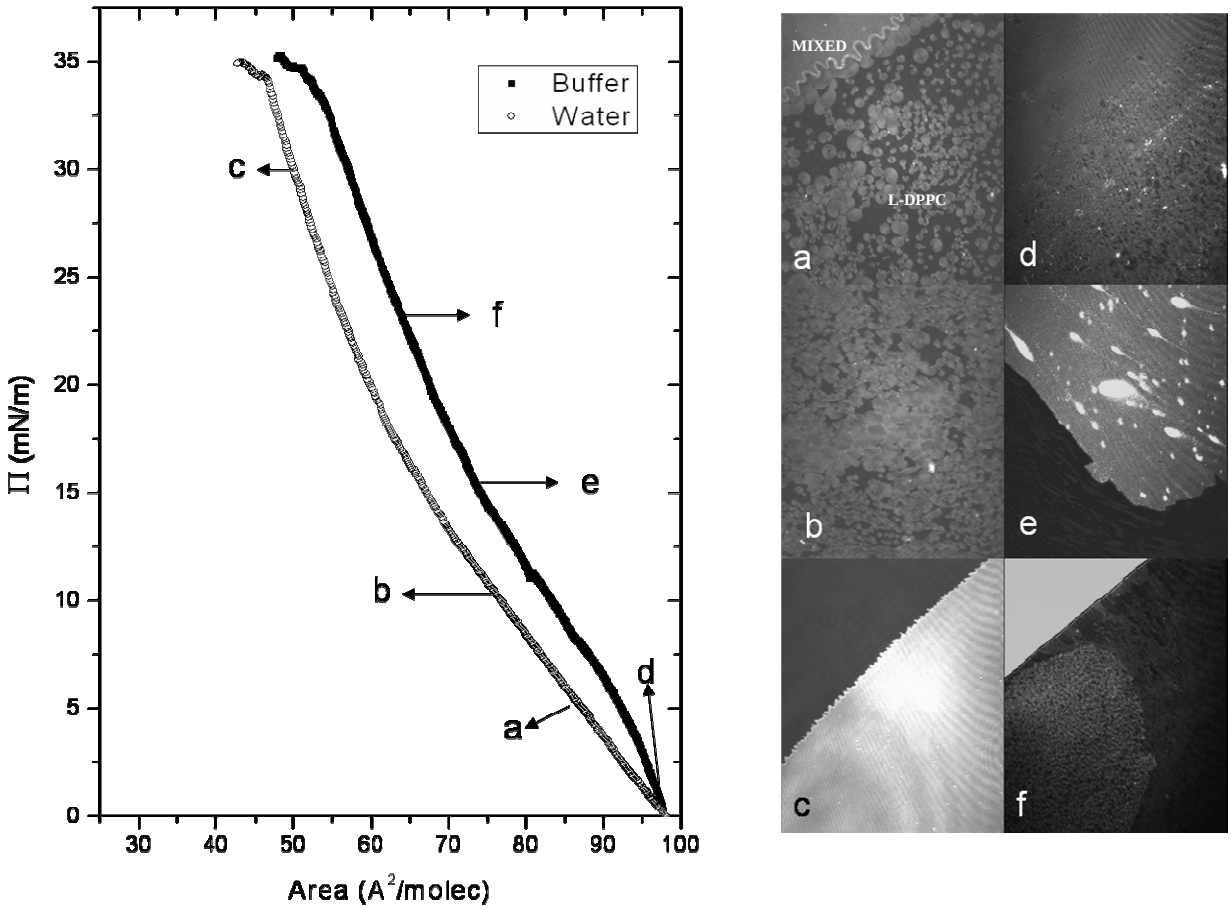

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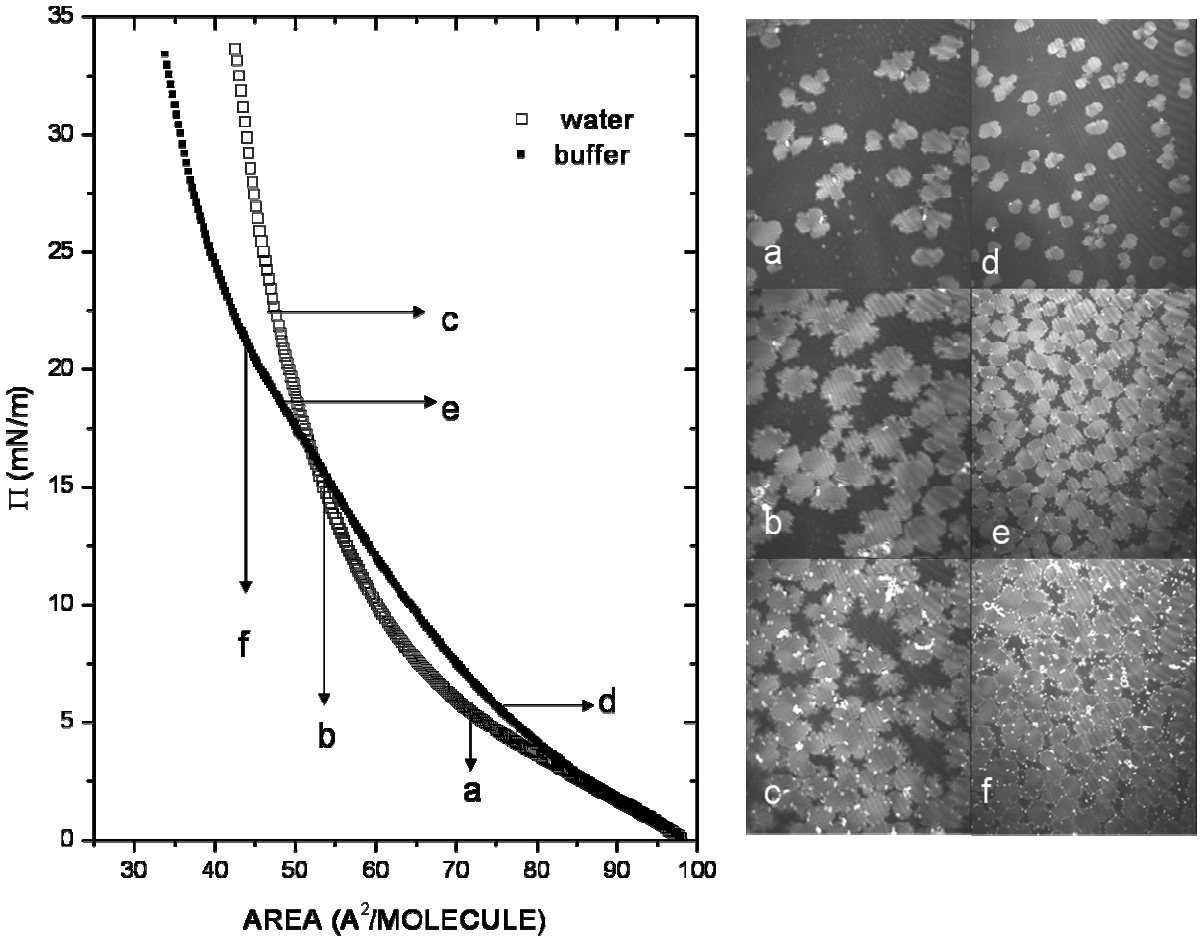

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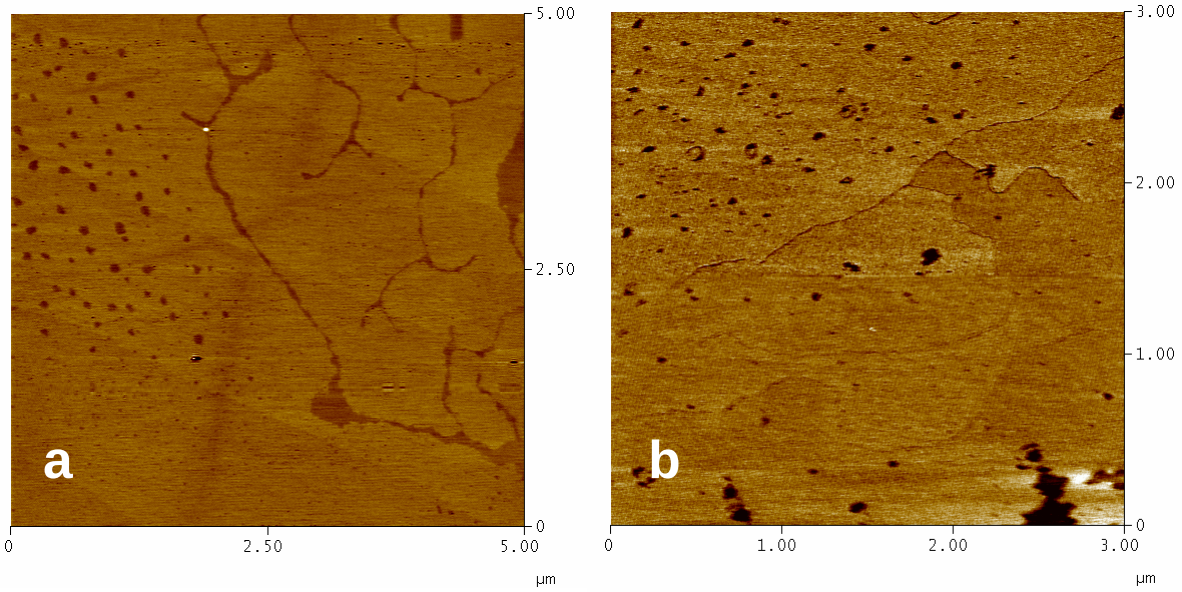
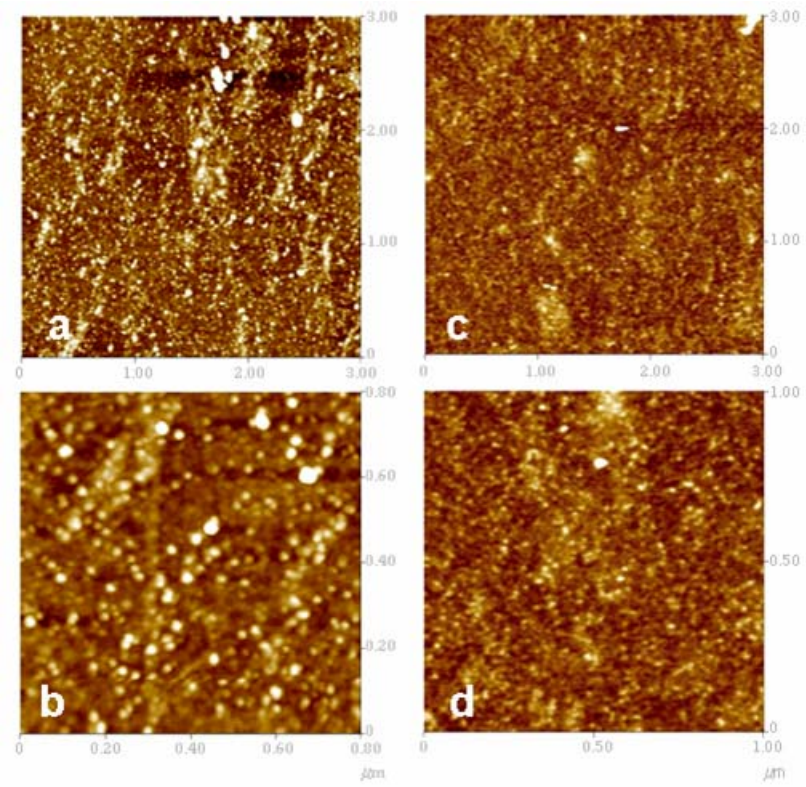


Figure 6. Garcia-Gonzalez *et. al.*



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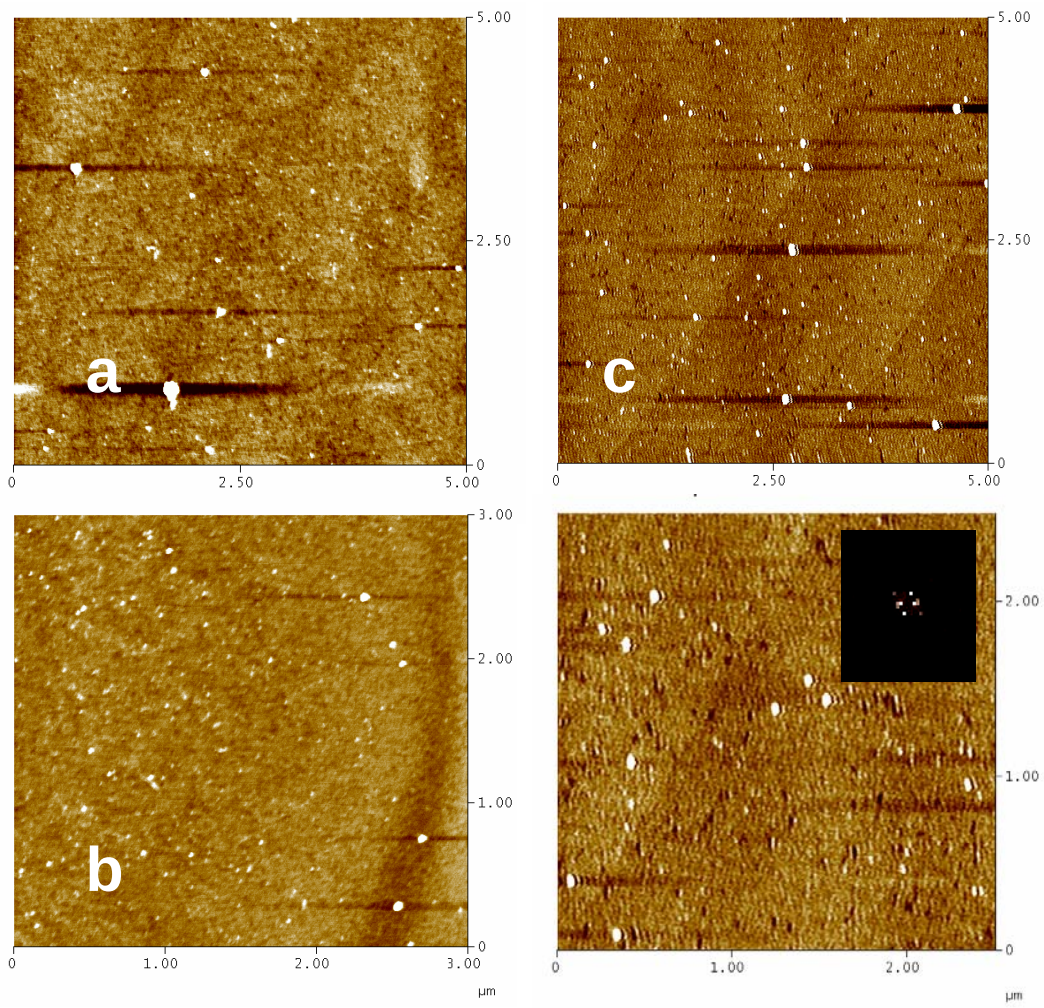

Figure 8. Garcia-Gonzalez *et. al.*

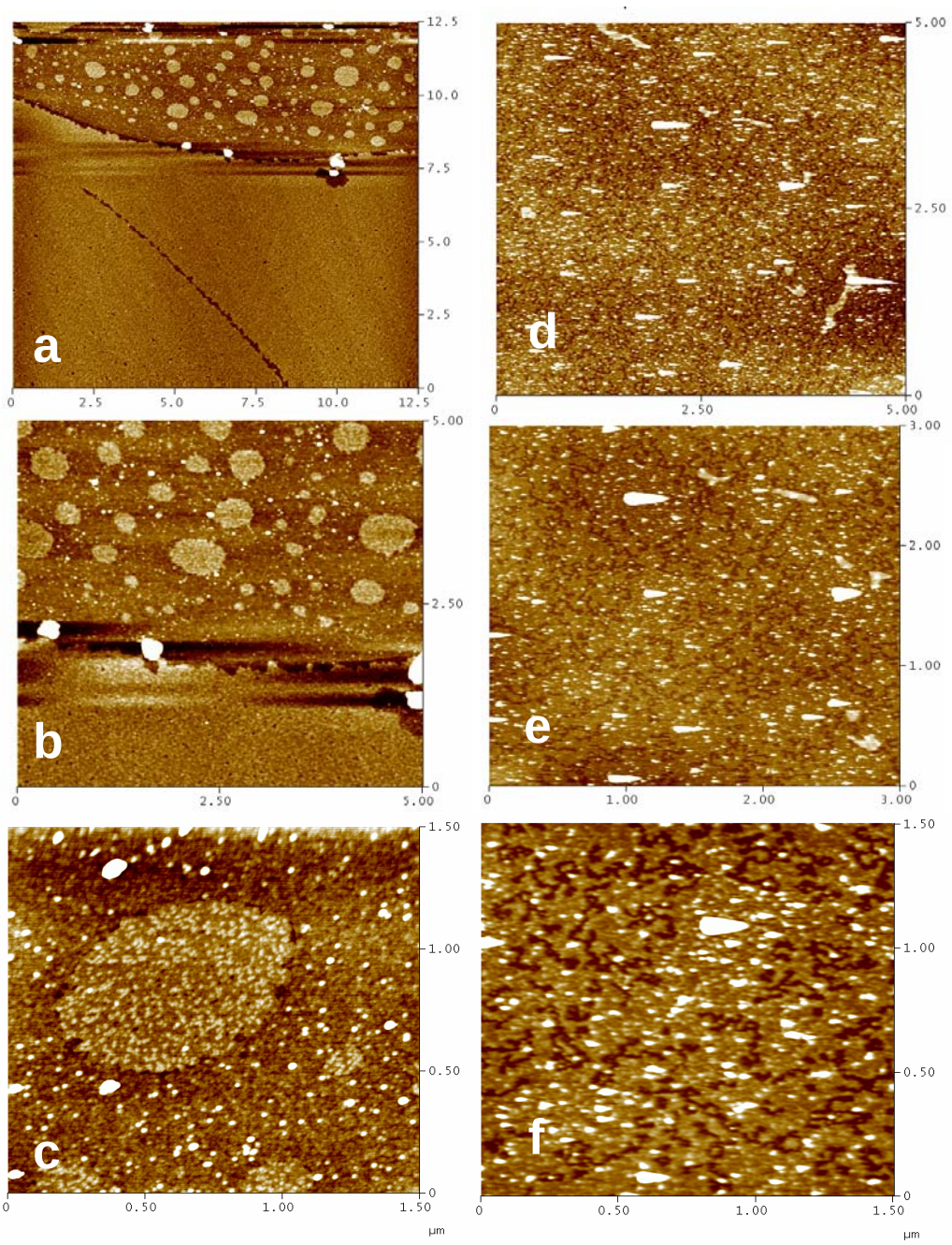

Figure 9. Garcia-Gonzalez *et. al.*

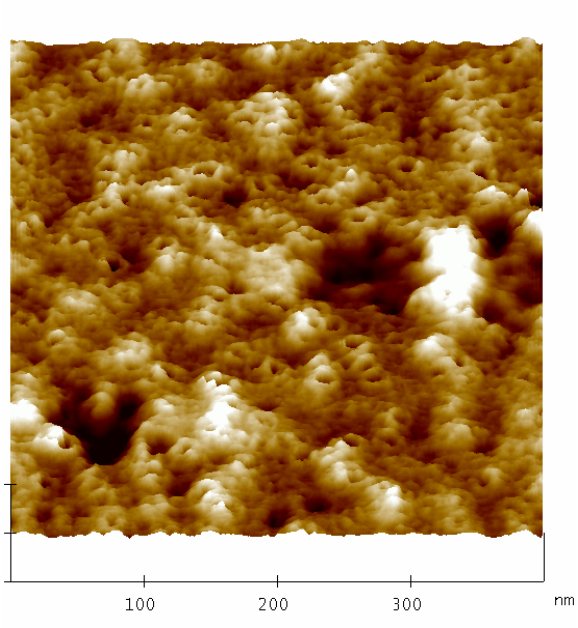

Figure 10. Garcia-Gonzalez *et. al.*

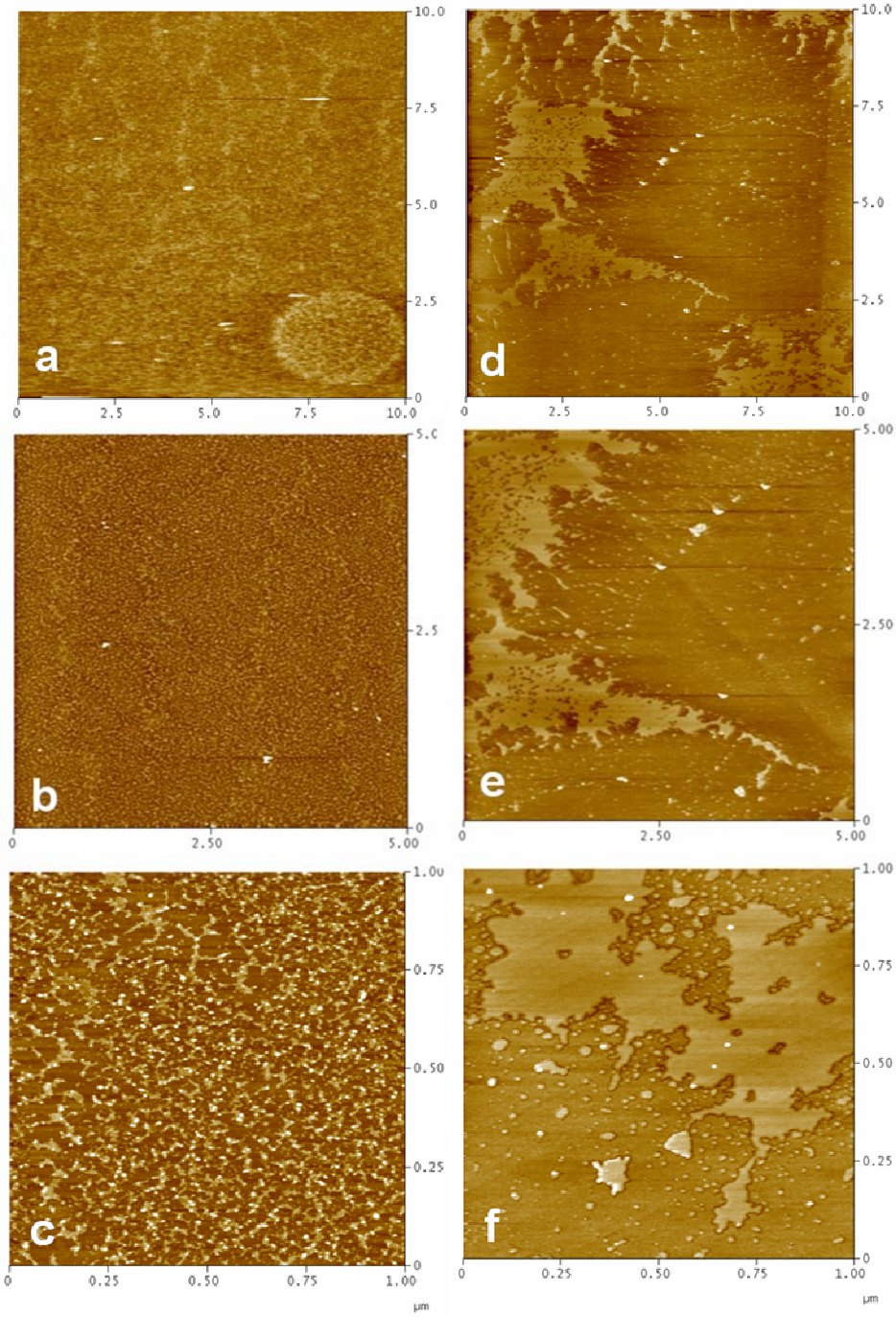

Figure 11. Garcia-Gonzalez *et. al.*