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LIPOSOME STABILITY VERIFICATION BY ATOMIC FORCE MICROSCOPY

E. F. de Souza[1] and O. Teschke[2]

[1] Faculdade de Química, CEATEC/PUC-Campinas, Rodovia Dom Pedro I, Km 136 – Parque das Universidades – 13086-900, Campinas, SP, Brasil
[2] IFGW/UNICAMP, Cidade Universitária Zeferino Vaz, 13083-970, Campinas, SP, Brasil

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Abstract. The observation of liposomes made up of phospholipides by atomic force microscopy has enabled direct visualization of their structure. Molecularly resolved force versus distance curves of the liposomes supported on silanized mica in water display the organization of hydrocarbon chains and the presence of distinct adsorbed structures. Multiple vesicle liposomes adsorbed on mica and observed within liquids and air show the same average diameter as the ones measured by photon correlation spectroscopy, indicating that their size was unchanged during the drying process. The as prepared liposomes are formed by smaller vesicles that aggregate to give the ~200 nm diameter vesicles.

1. INTRODUCTION

Liposomes are spherical, bilayer vesicles that form spontaneously when certain phospholipids are dispersed in water. Liposomes made up of phospholipids have been considered as both biomembrane models and as drug carriers. Various functions associated with biomembranes, such as aggregation, fusion and selective permeability, which depend on the hydrophilic-lipophilic balance of the contents of phospholipids must be taken into consideration when developing liposomes. Lipid vesicles also have various advantages as drug carriers, being biodegradable, having low toxicity and being able to encapsulate a hydrophilic substance within an aqueous environment and a lipophilic material within the lipid phase [1]. When they are applied to the field of drug delivery systems, a controlled release is one of the most important functions in the design of liposomes.

The aqueous dispersion of lipid vesicles are metastable systems, therefore, the study of the liposomes stability, aggregation or fusion is important throughout the various stages of their applications. Aggregation is then a critical characteristic behavior of liposome systems as it depends on the over-all interactive forces between lipid vesicles themselves and between the vesicles and the substrate [2]. Atomic force microscopy (AFM) offers the unique opportunity to probe local physical properties and interaction forces of lipid bilayers with nanoscale lateral resolution, thereby providing new insight into the molecular mechanisms of liposomes adhesion and membrane fusion. Thomson *et al.* [3] showed that liquid bilayer structures, partly fused and isolated liposomes can be observed by AFM. A broad spectrum of AFM applications have emerged in model membrane biophysics, allowing the study of the structure and function of biomembranes and biological processes such as molecular recognition, enzymatic activity and cell adhesion processes. Recently, Reviakine and Brisson [4] studied the early growth stages of supported zwitterionic phospholipid bilayers by vesicle fusion on mica. The topography change of liposomes from a vesicle shape to

Corresponding author: E.F. de Souza, e-mail souzaef@puc-campinas.edu.br

Table 1. Composition of the studied liposomes.

Sample	*PC*(mol)	*POPC* (mol)	*DPPC* (mol)	CH(mol)	*Phospholipid: cholesterol* (molar ratio)
PC/CH 1.75:1	$1.27 \cdot 10^{-4}$			$7.27 \cdot 10^{-5}$	1.75:1
POPC/CH 1.75:1		$1.30 \cdot 10^{-4}$		$7.42 \cdot 10^{-5}$	1.75:1
DPPC/CH 1.75:1			$1.42 \cdot 10^{-4}$	$8.10 \cdot 10^{-5}$	1.75:1
PC/CH 1.50:1	$1.11 \cdot 10^{-4}$			$7.40 \cdot 10^{-5}$	1.50:1
PC/CH 2.00:1	$1.48 \cdot 10^{-4}$			$7.40 \cdot 10^{-5}$	2.00:1

a bilayer through their direct adhesion on a mica surface was recorded in situ by AFM [5].

In this work we have investigated the structure of liposomes adsorbed on mica. Liposome/substrate interaction was modified by using surface treatment processes which render variable strength interactions. Adsorbed vesicles were initially observed on untreated mica and then on silanized mica substrates. Liposomes were also observed in water after drying liposome solutions on mica surfaces. The visualization of the adsorbed liposome allows us to determine if they are isolated in aqueous solution or clustered to form multiply aggregated structures.

2. EXPERIMENTAL

Materials. Egg yolk L-α-phosphatidylcholine 99% (PC), α-palmitoyl-γ-oleoyl-L-α-phosphatidylcholine 99% (POPC), dipalmitoyl-L-α-phosphatidylethonolamine 98% (DPPC) and cholesterol 99% (CH) were purchased from Aldrich. Analytical reagent grade chloroform and methanol were purchased from Merck. All chemicals were used without further purification. The water was distilled and deionized using a Milli-QУ System.

Preparation of the liposomes suspension. Suspensions of large vesicles were made by reverse-phase evaporation [6-8], using different proportions of L-α-phosphatidylcholine, β-palmitoyl-γ-oleoyl-L-α-phosphatidylcholine or dipalmitoyl-L-α-phosphatidyl-ethonolamine and cholesterol, according to Table 1. The desired quantities of phospholipid and cholesterol were dissolved in 150 mL of chloroform-methanol (2:1, v/v) and the solvents were evaporated to dryness on a rotary evaporator (Fisaton) to form a dry organic film. The system was kept under vacuum for at least 3 hours to remove the last traces of the organic solvents. A 150-mL volume of deionized water at 328K was added to the phospholipid-cholesterol mixture. This mixture was then sonicated for 10 min using a bath-type sonicator (Odontobras 1440D, 20 kHz). In order to remove the largest particles and hence obtain a more homogeneous liposome population, the suspension was extruded through cellulose acetate membranes of 0.45 μm pore size in a Millipore© filtration cell.

Atomic force microscopy. The AFM observations were performed with a TopoMetrix TMX2000© AFM. All the images were obtained with the contact mode using triangular cantilevers (Si_3N_4) which were 100 nm long and had a spring constant of 0.03 N/m. Moscovite mica was chosen as a solid substrate and used immediately after cleavage in a clean atmosphere. The initial surface (cleaved mica surface) characterization was performed in water by using a fluid cell [9]. During this characterization experiment, the probe and cantilever were immersed completely in the water solution. The liposome suspension on mica was dried from air (65% humidity) for three hours. Images were recorded in the constant-force mode using sharpened silicon nitride tips mounted on cantilevers with nominal force constants of 0.03 N/m. The scan angle was 90°. Force was kept at the lowest possible value by continuously adjusting the set point during imaging.

Silanization of the mica surface. Immediately after cleavage the mica samples were placed in a Petri dish previously filled with 10 mL of a 2% w/w aminopropylthiethoxysilane (APTES) solution in dry toluene. The solvent was evaporated at 60 °C and the Petri dish was transferred to a stove and kept at 120 °C for 3 hours.

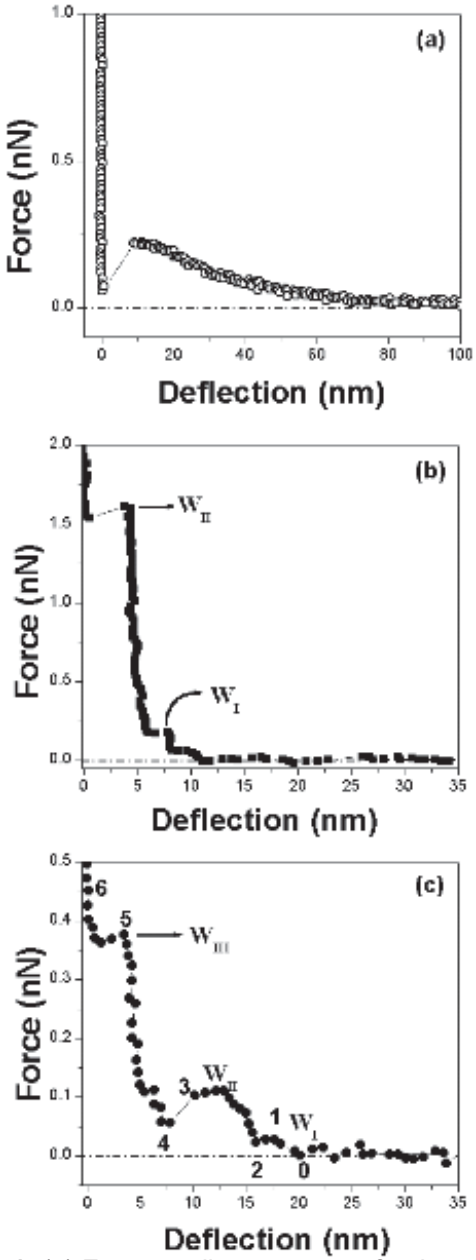

Fig. 1. (a) Force vs distance curve for the control experiment which was performed using mica substrates immersed in deionized water. (b) Force vs distance curve for mica surfaces immersed in a liposome solution. The repulsive deviation from horizontal line, (~10 nm from contact) is followed by an attractive regime, (indicated by W_I). A large repulsion component is measured when the tip further approaches the interface followed by an attractive regime (indicated by W_{II}, which corresponds to the thickness of a bilayer). (c) Force vs distance curve measured on a substrate dried from air in a 60% humidity atmosphere. Three bilayers are present in the tip/substrate interaction region: the first structure starting at 20 nm and ending at 15 nm, the second starting at 15 nm and ending at 8.5 nm and the third starting at 8.5 nm and ending at the substrate surface.

3. RESULTS AND DISCUSSION

Control experiment curves were performed using Si_3N_4 tips and mica substrates immersed in the Milli-Q Plus water and scanning mica surfaces. One of the typical force *vs* separation control curves is shown in Fig. 1a. No structure is observed in the force vs distance curve for distance close (~5 nm) to the mica/water interface.

Initially we tried to image liposomes PC/CH 1.75:1 on mica surfaces immersed in a liposome solution by scanning various regions of 1x1 cm^2 sample substrates. The scanning of the mica surface after an immersion period of 60 minutes does not show any adsorbed liposome. Force vs distance curves were then measured for this surface (Fig. 1b). Results show an uniformly covered surface. The repulsion deviation from the horizontal line, starting at ~10 nm from contact, is followed by an attraction regime, when the force achieves ~0.2 nN (indicated by W_I). A large repulsion component is observed when the tip further approaches the interface which is followed by an attraction regime (indicated by W_{II} at ~4 nm, which corresponds to the thickness of a bilayer). Fig. 1b then shows that in liposome solutions there are two attraction component regions indicated by W_I and W_{II} in the measured force vs distance curves. The first attraction is due to the compression and removal of phospholipids adsorbed from the tip surface and the second caused by the removal of a adsorbed phospholipid bilayer on the mica surface. The second removal structure results from the strong liposome/mica interfacial charge interaction; which causes the rupture of the liposome spherical structure and the formation of bilayers which spreads over the surface.

In order to decrease the interaction between the substrate and the liposomes in solution silanized mica surfaces were used as substrates and the observations were also undertaken in aqueous environment. The substrate and liposome interaction were still too strong, resulting in the rupture of the liposome and the formation of bilayers absorbed on the mica surface. Therefore we decided that before scanning the sample, the solution deposited on the substrate should be dried from air in a 60% humidity atmosphere. The reason for this extra step in the liposome fixation process is that during evaporation the decrease in the water volume present in the liposome/substrate interaction region results in an increase in the counter-ion concentration and consequently a decrease in the double-layer width. When contact is established between the substrate and the liposome, both surfaces are approximately

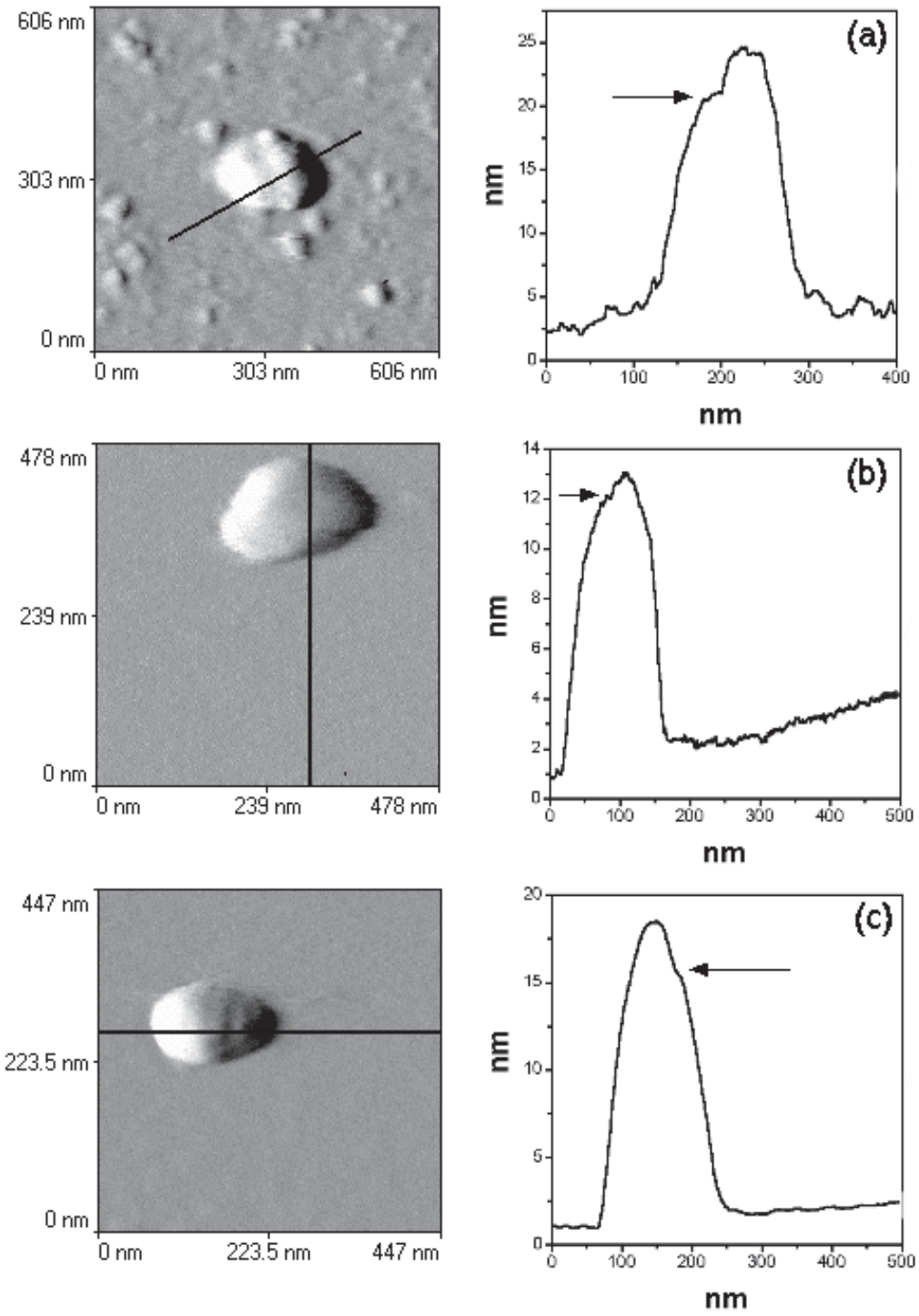

Fig. 2. Images and vertical profiles of liposomes adsorbed on a mica surface from aqueous solutions dried for observation. (a) Shows a typical image and the corresponding vertical profile of the same liposome for the PC/CH 1.75:1 sample. (b) Shows the same for the POPC/CH 1.75:1 sample. (c) Shows the same for the DPPC/CH 1.75:1 sample. (c) Shows the same for the PC/CH 1.50:1 sample. (e) Shows the same for the PC/CH 2.00:1 sample. The arrows in the vertical profiles of the liposomes indicate the presence (or absence in the case of the liposome PC/CH 1.5:1) of smaller aggregated vesicles.

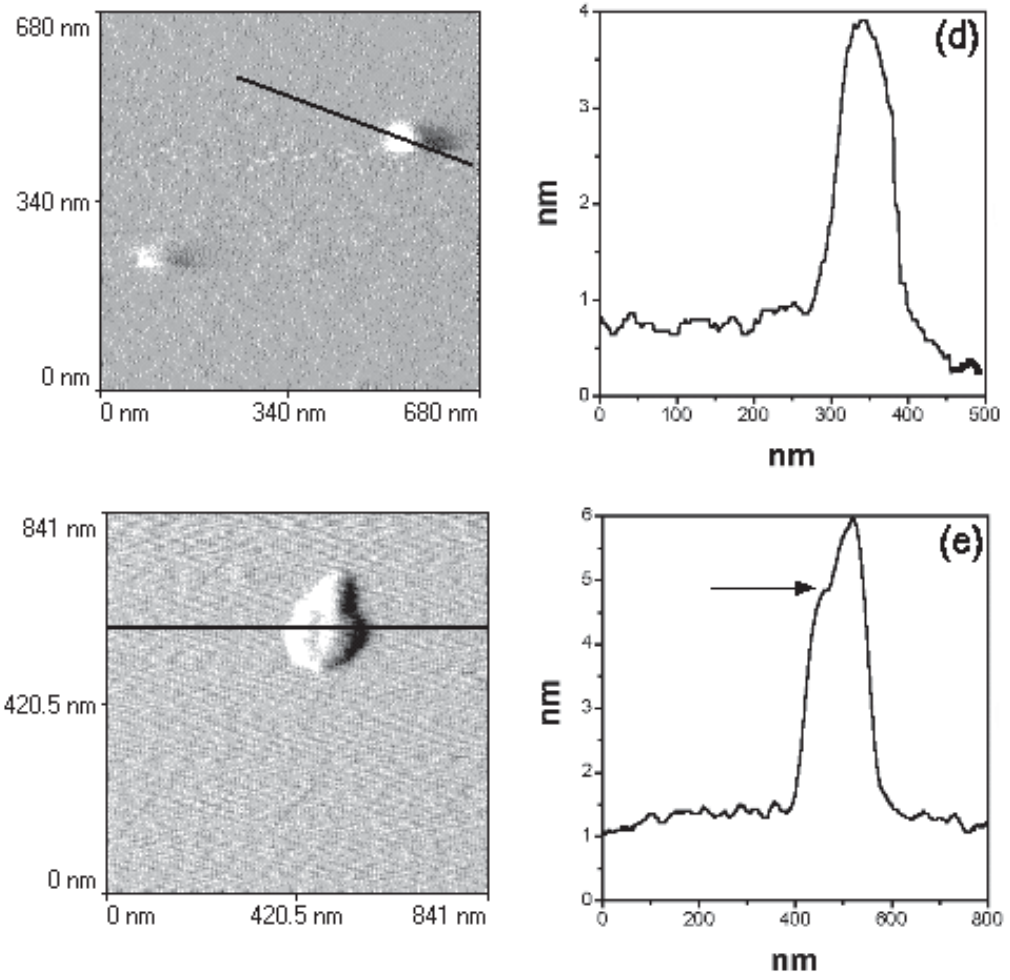

Fig. 2. Continued.

neutral and the electrostatic interaction is substantially decreased. The deposited structure thickness was then measured in water using force vs distance curves. Three repulsions and followed by attraction as previously described [10], are observed in the force vs separation curves shown in Fig. 1c: the first structure starts at 20 nm and ends at 15 nm, the second starts at 15 nm and ends at 8.5 nm and the third starts at 8.5 nm and ends at the substrate surface. The first region in the force vs distance curve (indicated by 0-2) corresponds to the compression (0-1) and removal (1-2) of the adsorbed layer from the tip surface. The second measured structure in the interaction region (indicated by 2-4) starts at 15 nm from the interface; this structure is totally removed from the interaction region for a tip applied force of ~0.1 nN. The third bilayer present (indicated by 5-6) in the tip/substrate interaction region is only removed from this region for a tip-applied force of ~0.35 nN.

Samples dried from air show a smaller adsorbed bilayer rupture force than the force measured on wet (not dried) samples (compare Fig. 1c with Fig. 1b). This is related to the neutralization of surface charges at the liposome and substrate surfaces during the drying process.

In regions 2-3 and 4-5 shown in Fig. 1c, the liposomes respond elastically to small stress but undergo plastic deformation when stresses are severe (close to points 3 and 5). The hydrocarbon chains provide the membrane with considerable flexibility when liposomes are stressed. The elastic contribution during the compression of the top bilayer is indicated by 2-3 in Fig. 1c and it starts at 16 nm from the interface. The bilayer and the water inside the liposome are compressed up to the tip/substrate distance of 11 nm when the top bilayer is ruptured. The tip is then attracted to the region inside the vesicle. The range of attraction force is ~4 nm which corresponds to the bilayer width. In the

tip substrate interaction region, only the bilayer is left adsorbed at the mica/solution interface; this layer is compressed from 7 nm up to 3.8 nm when there is the rupture of the membrane and the tip is attracted to the mica surface. The rupture force necessary to perforate the bilayer adsorbed at the mica interface is approximately three times larger than the rupture force of first bilayer perforated by the tip, which corresponds to the top of the adsorbed liposome starting at 16 nm away from the interface.

The rupture force (indicated by 1) of the bilayer adsorbed on an uncharged tip is equal to ~0.02 nN. The rupture force of the top bilayer of the liposome is ~0.1 nN and finally the one corresponding to the adsorbed bilayer on the mica is ~0.35 nN. The rupture force decreases as the bilayers are further apart from the mica substrate. A substantial part of the rupture force is then associated with the electric field generated by the mica interfacial charges that decays exponentially as it separates from the interface [11].

Liposomes formed by other PC/CH compositions were then imaged. Fig. 2a shows a typical image of liposomes PC/CH 1.75:1, Fig. 2b of liposomes POPC/CH 1.75:1, Fig. 2c of liposomes DPPC/CH 1.75:1, Fig. 2d of liposomes PC/CH 1.50:1 and Fig. 2e of liposomes PC/CH 2.00:1 adsorbed in silanized mica and then dried. Except for the liposomes PC/CH 1.50:1, the vesicles measured dimensions are of 210±40 nm suggesting that the POPC and DPPC phospholipids substituting groups (palmitoyl and oleoyl) do not change significantly the vesicle size. Moreover, the images suggest that these liposomes are formed by the aggregation of smaller vesicles indicated by the steps observed in the corresponding vertical profile image.

The situation is quite different for the case of the liposomes PC/CH 1.50:1 shown in Fig. 2d which show images that corresponds to spherical vesicles and display diameters of 90±10 nm. This observations is in agreement with the report that the incorporation of cholesterol into phospholipid bilayers affects the interaction forces between lipid vesicles decreasing the van der Waals attraction and increasing the net repulsion forces between bilayers, thus also increasing their stability with respect to the aggregation and fusion [2,12]. Thus the reduction of the PC:CH ratio (augmentation of the cholesterol content in the lipid bilayer) seems effectively to avoid the fusion of the small vesicles observed for the other PC:CH liposomes studied.

We decided then to investigate the structure of the aggregated liposomes PC/CH 1.75:1 by observing dried samples deposited in mica and then

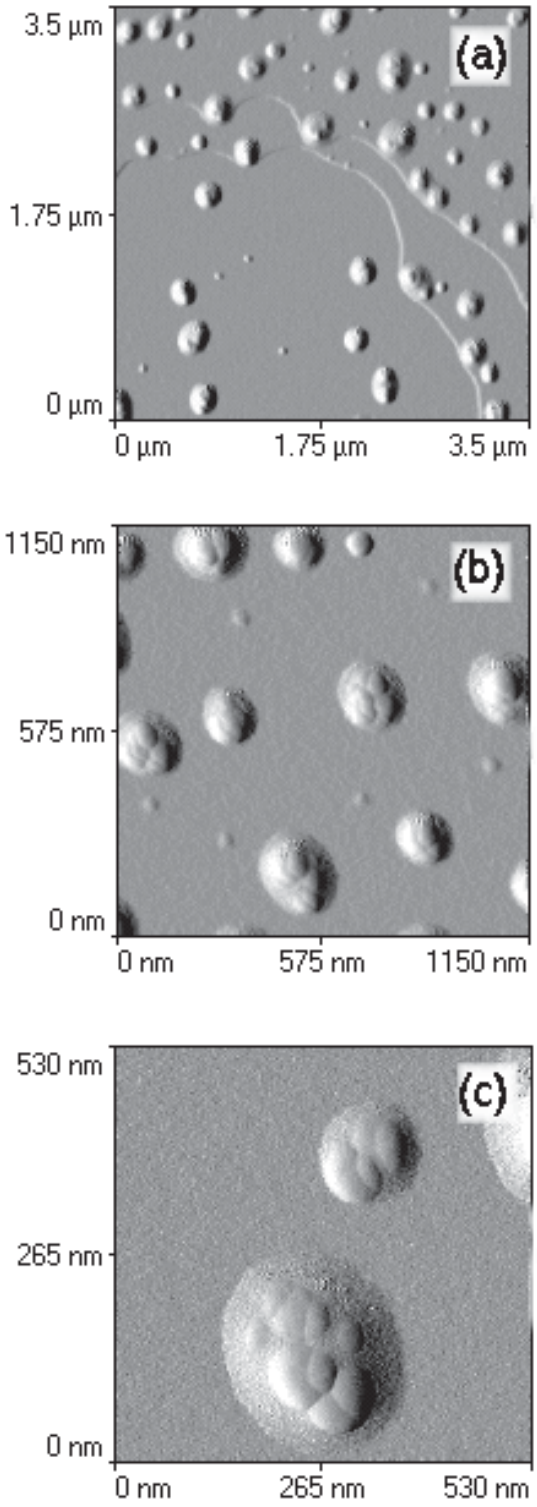

Fig. 3. Liposome adsorbed on mica from an aqueous suspension dried previous to observation from air. (a), (b) and (c) show successively larger amplifications. Some of spherical shaped vesicles ruptured spontaneously and were deformed to produce a flat supportive film seen at the right side of Fig. 3a. Deposited on these bilayers flattened structures there are 50 nm to 220 nm diameter liposomes, most of then formed by the aggregation of multiple vesicles .

scanned in air. The sample was prepared by the depositing a 3 mL volume of liposome dispersion on a 1 cm^2 area of a mica surface. An overview of the dried structure is depicted in Figs. 3a, 3b and 3c, showing successively larger amplifications. Some of almost spherical shaped vesicles ruptured spontaneously and were deformed to produce a flat supportive film on the mica surface (Fig. 3a). Most of the observed liposomes in Fig. 3 show a structure that has an outer deformed layer probably associated with a flattened spherical shaped vesicle and central vesicles that did not rupture. The most common structure corresponds to a liposome formed by the aggregation of multiple vesicles. Aggregation meaning the formation of a larger vesicle from two or more smaller vesicles, i.e., refers to joining of two or more smaller vesicles.

We propose the following formation mechanism for the deposited liposomes on the mica surface: during the adsorption process the liposome formed by the aggregation of various vesicles has the lower part of its structure in contact with the mica substrate. Due to mica/liposome interaction this structure is ruptured forming a flattened bilayer that adheres to the surface. Since most of the liposome are formed by the aggregation of multiple vesicles the contact liposome/substrate structure is formed by a flat bilayer; the other aggregated vesicles of the liposome are shown at the top as flattened spherical shaped vesicles.

The average size of the liposomes PC/CH 1.75:1 in solution measured by photon correlation spectroscopy is ~200 nm. Starting with a population of 200 nm mean diameter liposomes in solution, aggregation during the drying process would result in larger diameter sized liposomes. However, these larger diameter liposomes were not observed; therefore, probably most of the aggregation process takes place during the preparation of the liposomes and not during the drying process where the substrate/liposome interaction is probably stronger than the liposome/liposome interaction.

Except for the case of the liposomes with low phopholipid/cholesterol ratio, the dispersions of liposomes described in this work are then formed by multiple vesicles and this aggregated structure visualization is only possible by AFM imaging because the aggregated vesicles size forming the liposome is typically ~20-80 nm. The aggregated liposome structure has to be considered when developing liposomes is applied to the field of cosmetics, food additives and drug delivery systems since the selective permeability is substantially modified in aggregated vesicles when compared to single spherical ones.

4. CONCLUSIONS

High resolution AFM has enabled direct visualization of liposome supported on mica, in air and silanized mica in an aqueous media resolving multiple aggregated vesicles forming the liposome. The average sized liposome imaged by AFM shows that depending on the phopholipid/cholesterol ratio the liposomes are formed by the aggregation of a few vesicles or by isolated vesicles. Our results also show that when liposomes interact with a charged substrate (like mica) the structure formed by aggregation of various vesicles has a greater stability (understood as resistance to deforming their shape) than the one formed by a single spherical vesicle; consequently, there is greater resistance to rupture that modifies its function as a carrier of cosmetics, food additives and drugs.

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