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Specific Adhesion and Lipid Exchange between Complementary Vesicle and Supported or Langmuir Film

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The interaction between complementary vesicle and a supported or Langmuir monolayer containing either barbituric acid (BAR) or 2,4,6-triaminopyrimidine (TAP) derivatives has been investigated. The quartz crystal microbalance (QCM) technique permits one to detect vesicle adhesion and to quantify the process. The selectivity of the interaction and its dependence on various factors have been investigated: ionic strength, pH, concentration of recognition sites, and state of the supported membrane. Moreover, the Langmuir film balance technique has shown that lipid exchange and possibly hemifusion occur between the complementary vesicle and the Langmuir monolayer at the air/water interface. The results point to an electrostatic contribution to the vesicle adhesion, and they also support a picture according to which hydrogen bonding stabilizes the adhesion between complementary vesicles and supported membranes by a local arrangement of the complementary lipids in the contact area.

I. Introduction

Specific[1] recognition between membrane surfaces plays a key role in cell adhesion and in the control of tissue organization and growth. It may involve diverse mechanisms such as protein−substrate interactions, lipid diffusion, and structural changes in the membrane. Lipidic bilayers of vesicles represent a model of the cellular membrane, and the control of interactions between complementary vesicles should allow the generation of well-defined molecular systems possessing suitable recognition features and mimicking biological processes such as cell adhesion or fusion.[2] Molecular recognition between molecules and free membranes,[3] based on phosphate/guanidinium interaction, or between molecules and supported membranes,[4] involving hydrogen bonding and electrostatically complementary pairs, has already been shown to occur in water.[5,6] Our aim is to use such interactions for inducing specific adhesion or fusion between membranes. We have focused our research on the formation and the study of the interaction between decorated bilayers containing molecular recognition groups interacting by hydrogen bonding and electrostatic interactions. Previously one of our laboratories has investigated the interaction between vesicles presenting either electrostatic[7] or hydrogen bonding[8] complementarity. In the latter case it was found that complementary vesicles containing respectively TAP[1] and BAR lipids (Figure 1) interact selectively and undergo aggregation and fusion. Such a system represents a model of cell adhesion and is specially relevant for drug delivery. On the other hand it was also been shown that TAP and BAR lipids incorporated into monolayers at the air/water interface interact with complementary solutes in the subphase.[9]

Another approach consists of studying the specific interaction between vesicles and supported membrane, as was done for the biotin/streptavidin,[10] antigen−antibody,[11] or protein-receptor association.[12] Vesicle adhesion has been investigated by near infrared surface plasmon resonance (NIR−SPR) for vesicles reacting chemically with supported membrane[13] and by reflection interference contrast microscopy (RICM) either for biotin-containing vesicles that adhere to biotin-functionalized supported membrane via streptavidin or for electrostatically interacting vesicles.[14] Furthermore, the quartz crystal microbalance (QCM) technique, which detects the adsorption of molecules onto a substrate by measuring in situ the mass change, has already been developed by investigating the binding of molecules to a supported lipid layer via hydrogen bonding.[15]

Here we report a study of the molecular recognition between vesicle and monolayer or multilayer via complementary TAP and BAR lipids involving hydrogen bonding and electrostatic interactions, employing the QCM tech-

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(1) Abbreviations: BAR, barbituric acid lipid; EPC, egg lecithin; EO_4, alcohol lipid; LC, liquid condensed; LE, liquid expanded; $QCM(TAP)_n$, quartz crystal microbalance bearing in layers of TAP LB film; TAP, 2,4,6-triaminopyrimidine lipid.

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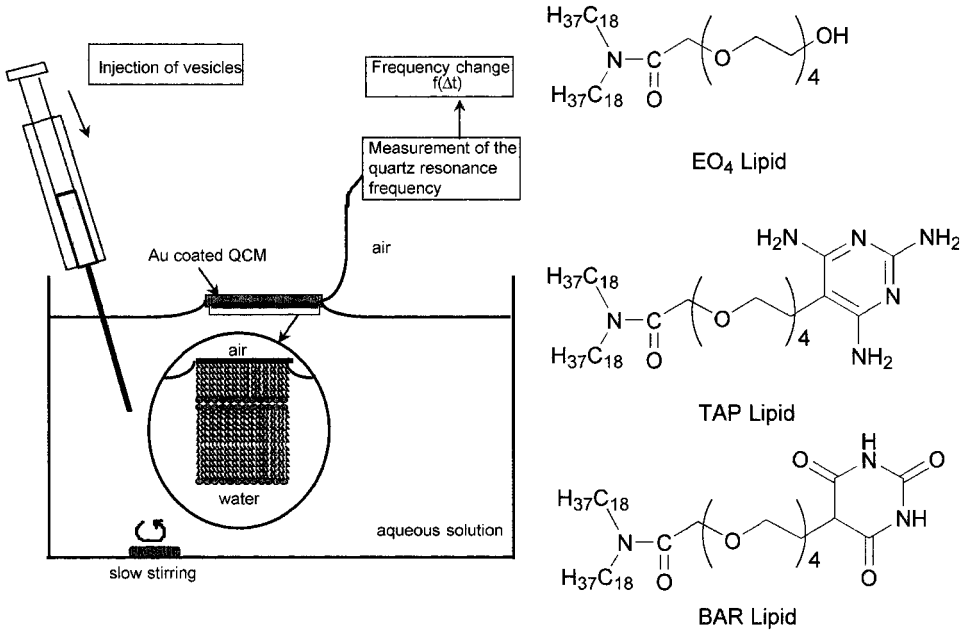

Figure 1. Schematic illustration of the experimental setup for investigating vesicle adhesion to a quartz crystal microbalance (QCM) bearing a lipid multilayer in aqueous solution and chemical structures of the lipids used.

nique to detect vesicle adsorption onto supported multilayer through in situ mass change in aqueous solution. This technique provides a powerful and simple tool for quantifying vesicle adhesion and investigating the nature of the interaction. Furthermore, we have also studied the interaction between vesicle and complementary Langmuir monolayer and compared the results with those obtained from QCM experiments. These two methods give complementary information on vesicle/monolayer interaction and provide very useful tools to investigate the mechanism of vesicle adhesion and hemifusion to a 2-dimensional polymolecular assembly.

II. Experimental Section

A. Materials. Egg lecithin (EPC) was used as purchased from Sigma. The preparation method of the synthetic amphiphiles is briefly described in our previous paper[7] and will be detailed elsewhere. The structure and the purity of the TAP, BAR, and EO_4 lipids were confirmed by elemental analyses and by ^1H and ^{13}C NMR, as well as mass spectroscopies. The elemental analyses of the synthesized lipids gave the following results. Calcd for TAP lipid ($C_{50}H_{98}N_6O_5$; $M = 863.36$ g·mol^{-1}): C, 69.56; H, 11.44; N, 9.73. Found: C, 68.91; H, 11.46; N, 9.09. Calcd for BAR lipid ($C_{50}H_{94}N_3O_8Na{\cdot}H_2O$ M = 906.31 g·mol^{-1}): C, 66.26; H, 10.68; N, 4.64. Found: C, 66.69; H, 10.58; N, 4.76. Calcd for EO_4 lipid ($C_{46}H_{93}NO_6$, $M = 756.24$ g·mol^{-1}): C, 73.06; H, 12.39; N, 1.85. Found: C, 72.95; H, 12.03; N, 1.88. The Britton-Robinson buffers at pH 4, 5, 7, and 9 and at an ionic strength of 0.1 M were prepared according to the literature.[16] The temperatures and latent heats of the gel transition for suspensions of TAP/EO_4 mixtures in water were investigated by differential scanning calorimetry (DSC) using a DSC-7 apparatus operated at 5 °C·min^{-1} under nitrogen.

B. Vesicle preparation. Vesicles were prepared according to reported procedures.[17]

Detergent dialysis method (DD).[18] A chloroform solution of lipid components (6.6 μmol) was evaporated under vacuum in a 10-mL round-bottomed flask at room temperature. A 10 molar equiv sample of *n*-octyl-β-glucopyranoside and 1 mL of aqueous solution were added. The solution was then dialyzed 3-fold against 1 L of pure water. The obtained vesicle suspension was filtered on a gel exclusion column (PD-10 column, Pharmacia). The final lipid concentration was 2.6 mM.

Sonication.[19] The chloroform solution of lipids was evaporated under vacuum at room temperature to produce a thin film on the wall of a 10-mL round-bottomed flask. After the addition of 2.5 mL of aqueous solution, the suspension was continuously sonicated with a Tomy ultrasonic disruptor UD-201 for 10 min (output 6) by cooling the flask at 0 °C. The final lipid concentration was 2.6 mM.

C. Quartz Crystal Microbalance Experiments. QCM Electrode. The QCM electrode employed is a commercially available 9 MHz, Au-coated quartz crystal, purchased from USI System, Fukuoka, Japan. One side of the QCM was covered with a rubber case to avoid contact with the solution.

***π*−*A* Isotherms and Monolayer Deposition on QCM.** *π*−*A* isotherms were measured with a computer-controlled film balance system FSD-50 (USI System, Fukuoka, Japan). A mixture of benzene/ethanol (80/20) was used as a spreading solvent. Compression was started about 10 min after spreading at a rate of 55 mm^2·s^{-1}. The subphase temperature was kept at 20 ± 0.2 °C. The surface pressures were measured by a Wilhemy plate, which was calibrated with the transition pressure of an octadecanoic acid monolayer.

The monolayers were transferred at 50 mN·m^{-1} onto an Au-coated QCM electrode first by the vertical dipping method with a dipping speed of 20 mm·min^{-1} (down stroke) and 5 mm·min^{-1} (up stroke). Transfer ratios were almost unity (Y transfer type). After two monolayers were transferred by this method, a third monolayer was attached horizontally to the Langmuir film at a surface

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pressure of 50 mN·m^{-1} (excepted in the case of the study with various pressures). Then the QCM electrode was maintained in solution to avoid any change in the deposited multilayer.

Frequency Change Measurements. The QCM with the deposited film was dipped in a 10 mL-glass recipient and the solution was stirred slowly in order to suppress the effect of the diffusion process of vesicles. The stirring did not affect the stability of the supported multilayer. The QCM was connected to a frequency generator for driving the quartz at its resonance frequency. The frequency changes were followed by a frequency counter (Iwatsu, model SC 7101) attached to the microcomputer system (NEC, Tokyo, model PC 9801). When the 9 MHz-QCM modified with the rubber was introduced in a stirred aqueous solution, the frequency decreased by about 2350 ± 50 Hz, which is in good agreement with the shift due to the environment change mentioned in the literature.[14] After three monolayers were deposited and equilibration took place, the frequency of the coated QCM decreased by about 210 ± 10 Hz. The system exhibited a very good reproducibility in the frequency shifts caused by dipping the QCM in the solution and by depositing the multilayer. One can estimate the quantity of lipid deposited from the Sauerbrey equation[20]

$$\Delta f = \left(- \frac{2 f_0^{\,2}}{(\mu_q \rho_q)^{1/2}} \right) \frac{\Delta m}{A} \qquad (1)$$

where ΔF is the change in resonance frequency, resulting from a change in mass Δm, f_0 is the resonance frequency of the quartz (9×10^6 Hz), μ_q is the shear modulus of the quartz (2.947×10^{13} g·m^{-1}·s^{-2}), ρ_q is the density of the quartz (2.648×10^6 g·m^{-3}) and A is the apparent area of the QCM. By taking into account the characteristics of the quartz resonators used, one obtains the following relationship between adsorbed mass and frequency shift:

$$\Delta f = (-1.83 \times 10^4) \frac{\Delta m}{A} \qquad (2)$$

Here Δm is the mass change (in g) and A is the apparent area of the QCM (1.6×10^{-5} m^2) and the roughness of the surface is neglected.

The frequency shift produced by the deposition of three monolayers (210 ± 10 Hz) is in agreement with the deposition of three lipid monolayer with a 40 Å^2 mean molecular area (one monolayer corresponds to 56 ng) if one assumes that the transfer ratio is 1. One finds that 1 Hz change in frequency corresponds to 0.9 ng. Furthermore, a relationship between the frequency shift and the thickness e (in Å) of the deposited material can be deduced from (2)

$$\Delta f = (-1.83 \times 10^{10}) \rho e \qquad (3)$$

where ρ (in g·cm^{-3}) is the density of the deposited material.

The stability of the frequency with time was checked and the frequency increase observed before the addition of vesicles is slow enough to be neglected in the course of the experiment. All the experiments were carried out in a temperature-controlled room at 20 °C.

D. Surface Pressure Measurements. The surface pressures were measured by a Wilhemy plate in a temperature controlled room at 20 °C. Langmuir films are made in a 10 mL small glass beaker (constant area: 9.62 cm^2) containing 5 mL of pure water. A mixture of benzene/ethanol (80/20) was used as spreading solvent. A vesicle suspension (500 μL, 2.6 mM) is injected in the subphase with a Hamilton syringe. The time course of surface pressure changes ($\Delta\pi$), responding to the injection of vesicles, was collected at a constant total area of the monolayer. The solution was not stirred to avoid perturbation in surface pressure measurements.

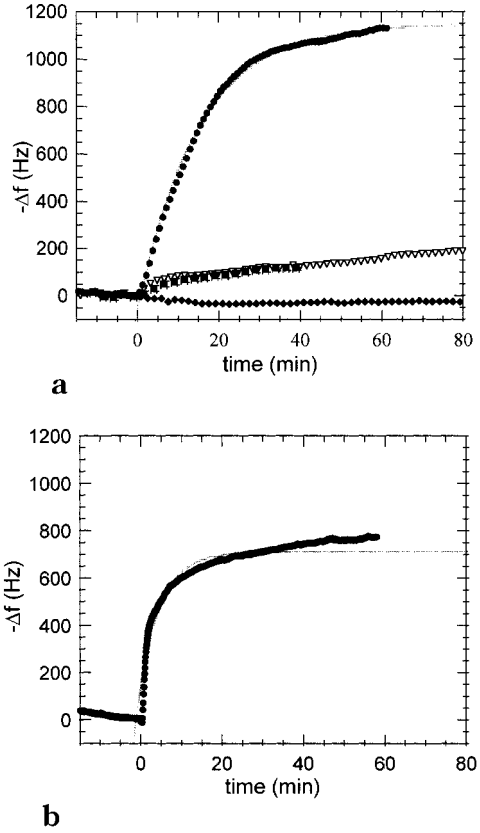

Figure 2. (a) Frequency changes in time of the QCM bearing three layers of TAP film (QCM(TAP)$_3$) after the addition of vesicles (0.3 mM total lipid concentration after addition) into the pure water subphase: (●) EPC:BAR (9:1) vesicles and (—) monoexponential fit; (■) EPC vesicles; (▽) EPC:TAP:BAR (90:5:5) vesicles; (◆) EPC:TAP (9:1) vesicles. (b) Frequency changes in time of the QCM(BAR)$_3$ film in pure water after the addition of EPC:TAP (9:1) vesicles (0.3 mM total lipid concentration after addition) and (—) monoexponential fit.

III. Results

A. Selective Interaction between Vesicles and LB Multilayers. The experimental setup shown in Figure 1 was used to investigate the selective interaction between complementary pairs of vesicles and LB multilayers. All the following experiments were carried out with a large excess of vesicles with respect to the total amount of lipids deposited on QCM. Indeed the molar ratio of recognition lipids within vesicles and within supported mulitlayer is around 1000. The frequency changes Δf as a function of time of the QCM deposited with three monolayers of TAP in pure water after the injection of various vesicles are shown in Figure 2a. The addition of complementary BAR vesicles in the solution induced a large change in quartz frequency (around 1000 Hz) within 30 min before reaching a plateau corresponding to the saturation of the surface.[21] On the other hand the addition of noncomplementary vesicles caused only a small change in quartz frequency

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(21) This behavior was observed whatever the method of vesicle preparation (dialysis detergent or sonication).

Table 1. Summary of the QCM Experiments Conditions and the Kinetics Values Evaluated from a Monoexponential Fit of the Curve $\Delta f(t)$[a]

expt	(lipid multilayer)$_n$	vesicle	π (mN·m^{-1})	subphase	τ (min)	Δf_{eq} (Hz)	k_d (min^{-1})
1	$(TAP)_3$	BAR:EPC (1:9)	50	pure water	15	1150	*b*
2	−	TAP:EPC (1:9)	−	−	*c*		
3	−	BAR:TAP:EPC (5:5:90)	−	−	14	130	0.063
4	−	EPC	−	−	19	140	0.046
5	$(BAR)_3$	TAP:EPC (1:9)	50	pure water	4	670	0.104
6	−	BAR:EPC (1:9)	−	−	*c*	≈0	
7	$(TAP)_3$	BAR:EPC (1:9)	50	pH 4	8	690	0.050
8	−	−		pH 5	5	590	0.097
9	−	−	−	pH 7	5	510	0.111
10	−	−	−	pH 9	2	450	0.304
11	−	TAP:EPC (1:9)	−	pH 7	*c*	≈0	
12	$(TAP)_3$	BAR:EPC (1:9)	50	1 mM barbituric acid	6	110	0.151
13	$(TAP:EO_4)_3$(3:7)	−	−	−	2	270	0.382
14	$(TAP:EO_4)_3$(1:9)	BAR:EPC (1:9)	50	pure water	1	130	0.887
15	$(EO_4)_3$	−	−	−	*c*	≈0	
16	$(TAP)_1$	BAR:EPC (1:9)	50	pure water	15	1150	*b*
17	−	−	14	−	1	750	0.340
18	−	−	10	−	1	100	0.913

[a] Subscript *n* indicates the number of lipid layers in the film; − means that the value or the condition is the same than the previous lign; π is the transfer surface pressure; τ designates the time constant of the curve $\Delta f(t)$; Δf_{eq} is the final change in frequency corresponding to the plateau; k_d is the desorption constant calculated from Δf_{eq}, τ, and Δf_{max} following the eq 7. [b] Δf_{max} was taken as the Δf_{eq} value obtained in experiment 1. [c] These values were not measurables.

or none at all. Indeed no change in frequency was observed in the combination of TAP vesicles and a TAP multilayer. Concerning the EPC vesicles and the mixed EPC/BAR/TAP vesicles, the final frequency changes were around 200 Hz. To confirm the observed complementarity, we have carried out the symmetrical experiment by using TAP vesicles and a BAR multilayer. As shown in Figure 2b, the course of the frequency change in time exhibited a similar saturation behavior. The final value of frequency change was in the same range as that observed for the combination of BAR vesicles/TAP multilayer (around 800 Hz) but the saturation was faster.

The kinetics presented by all the curves can be fitted to a first-order rate equation characterized by the time constant τ and the final value of frequency change Δf_{eq} (see Table 1). It is important to note that the time constant τ of the phenomena decreases with Δf_{eq} in case of noncomplementary vesicles. Furthermore, the initial rate of frequency change was the same for any added vesicle suspensions, around 55 ± 5 Hz.min^{-1} corresponding to approximatively 6×10^6 vesicles/s.[22]

B. Nature of the Interaction between Vesicle and Supported Multilayer. A set of experiments was carried out using the QCM technique to investigate more precisely the nature of the interaction between vesicles and supported membranes.

Effects of Ionic Strength and pH on Vesicle Adhesion. Considering the pK_as of the 2,4,6-triaminopyrimidine TAP (6.8 in bulk for 5-monosubstitued TAP) and the barbituric BAR headgroups (4.1 in bulk for unsubstitued BAR),[23,24] the electrostatic attraction between the negatively charged BAR vesicles and the positively charged TAP multilayer should play an important role (see Figure 3).[25] We have studied the effect of ionic strength and pH on vesicle adhesion. As shown in Figure 4a, in the presence of a pH 7 buffer at a constant ionic strength 0.1 M, the addition of complementary

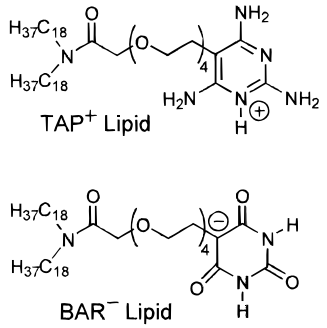

Figure 3. Protonated TAP^+ and deprotonated BAR^- lipids.

vesicles induces a weaker frequency change than in pure water. The final value of frequency change corresponding to the plateau (around 550 Hz) is smaller in the presence of the buffer. Furthermore, at a constant ionic strength of 0.1 M, the final frequency change remains significantly larger (by a factor of about 2) than that observed for noncomplementary vesicles (entries 2−4) at any pH, from 4 to 9, as shown in Table 1 (entries 7−11). Interestingly the surface coverage by vesicles at equilibrium increases as pH decreases.[26]

Competition between BAR in Vesicles and in Solution for Adhesion to a Supported Multilayer. We have carried out a competition experiment between BAR vesicles and barbituric acid molecules for a TAP multilayer. A QCM deposited with three layers of TAP monolayers was used on a 1 mM barbituric acid subphase. Figure 5 shows the frequency change in time of such a QCM after addition of a suspension of BAR vesicles in 1 mM aqueous barbituric acid. The interaction of BAR vesicles with the TAP supported membrane is completely inhibited in the presence of competing barbituric acid molecules in solution (see Table 1, entry 12).

Effect of the Surface Density of Recognition sites on Vesicle Adhesion. If the TAP/BAR hydrogen bond forms an extended "ribbon structure" as found in the solid state,[27,28] the density of the TAP recognition groups within

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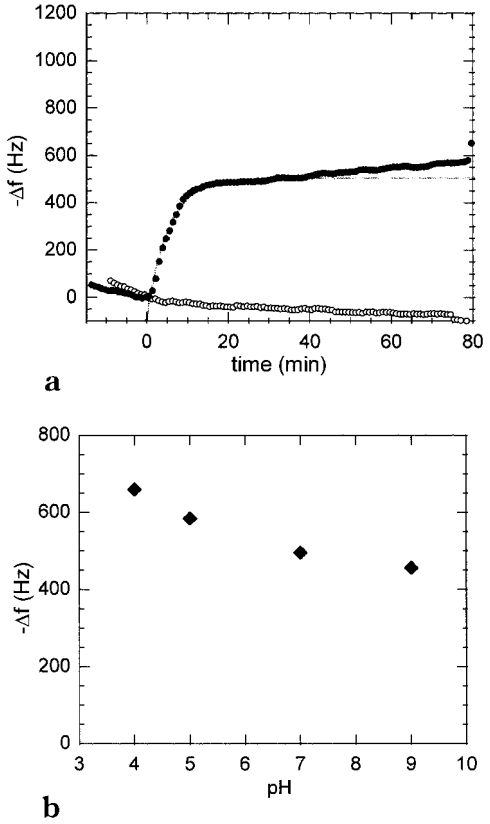

Figure 4. (a) Frequency changes in time of the QCM(TAP)$_3$ in pH 7 buffer (ionic strength 0.1 M) after the addition of vesicles (0.3 mM total lipid concentration after addition): (●) EPC:BAR (9:1) vesicles and (—) monoexponential fit; (○) EPC:TAP (9:1) vesicles. (b) pH effect on the final frequency change (Δf_{eq}) of the QCM(TAP)$_3$ after the addition of EPC:BAR (9:1) vesicles at constant ionic strength 0.1 M.

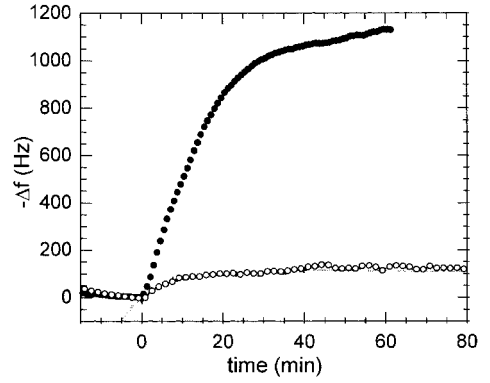

Figure 5. Competitive binding of barbituric acid solute and BAR vesicles to a TAP multilayer. Frequency changes in time of a QCM(TAP)$_3$ after the addition of EPC:BAR (9:1) vesicles: (●) in water; (○) in the presence of 1 mM barbituric acid in water and (—) monoexponential fit.

the multilayer should affect considerably its interaction with BAR. We have studied the adhesion of BAR vesicles to TAP/EO$_4$ multilayer films of different compositions to decrease the surface density of TAP sites within the supported film. First the $\pi-A$ isotherms were determined for mixed TAP/EO$_4$ monolayers. No significant change in the mean molecular area was observed when the fraction of TAP was varied as shown in Figure 6. Furthermore, we measured by DSC the temperatures and latent heats

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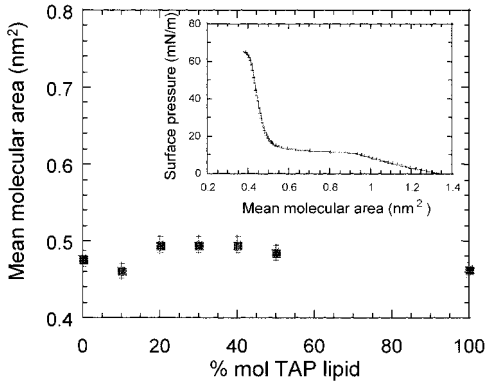

Figure 6. Mean molecular area of TAP/EO$_4$ mixture monolayers in the LC phase as a function of the molar percentage of TAP, deduced from the corresponding $\pi-A$ isotherms on a pure water subphase at 20 °C. Inset: $\pi-A$ isotherms of pure TAP monolayer on a pure water subphase at 20 °C.

Table 2. Transition Temperatures and Latent Heats of Aqueous Suspension of Mixed Sonicated Suspensions of TAP and EO$_4$ Lipids in Pure Water (total Lipid Concentration 12 mM)

TAP/EO$_4$ ratio	0/1	1/9	23/77	4/6
T_t (±0.2 °C)	43.8	43.9	43.9	43.9
ΔH_t (±1 kJ·mol^{-1})	19	23	24	26

of gel transition for suspensions of different TAP/EO$_4$ mixtures in water (see Table 2). The results concerning the adhesion of the BAR vesicles on QCMs bearing various TAP/EO$_4$ films are summarized in Table 1 (entries 13−15).

Effect of the State of the Monolayer Deposited on the QCM. All the previous experiments were performed with a supported multilayer transferred at 50 mN·m^{-1} in the liquid condensed (LC) phase. It is of interest to investigate whether the state of the transferred monolayer can modulate the interaction of the vesicles with the surface receptor QCM. This is another way for decreasing the surface density of TAP sites within the supported film. QCMs bearing TAP monolayers deposited at different surface pressures were prepared, and the frequency change caused by BAR vesicles was examined. No significant change in transfer ratio (almost 1) was detected by decreasing the transfer pressure from 50 to 10 mN·m^{-1}. No large heterogeneity was found by AFM and fluorescence microscopy in the case of theTAP LB films transferred at low transfer pressure.[9] The final frequency changes induced by vesicle addition at different transfer pressures are reported in Table 1 (entries 16-18). By comparing the molecular area and the final frequency change one sees that the vesicle behavior changes drastically at the phase transition. In the liquid expanded (LE) phase, the final frequency change (around 250 Hz) is much smaller than in the LC phase and the time required for reaching the plateau is also smaller.

C. Vesicles Interaction with a Langmuir Monolayer. In all the QCM experiments, lipid movements were very restricted within the crystallized supported multilayer so that lipid exchange between vesicles and supported membrane were strongly hindered. We have carried out a new set of experiments with another system for which lipid exchange is not inhibited. They involve the interaction between complementary vesicles and a Langmuir monolayer expanded on a constant interfacial area. In Figure 7a, the surface pressure change induced by BAR vesicle injection in the subphase is shown as a function of time. One observes a large increase in surface pressure in the case of a TAP monolayer until a plateau

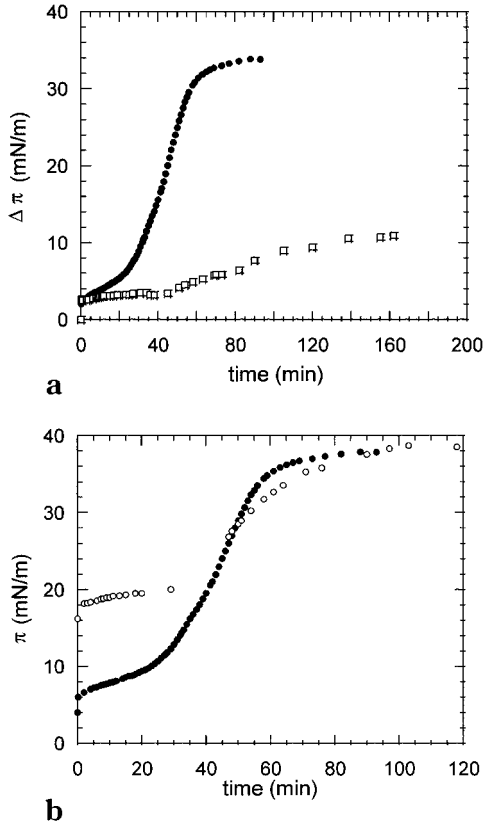

Figure 7. (a) Surface pressure changes of a TAP (●) or EO_4 (□) Langmuir monolayer after injection of EPC:BAR (9:1) vesicles in the subphase (0.3 mM total lipid concentration) at a constant mean molecular area 1.05 nm^2. (b) Effect of the mean molecular area on the surface pressure change of TAP Langmuir monolayer: (●) 1.05 nm^2; (○) 0.6 nm^2.

at 38 $mN \cdot m^{-1}$ is reached, whereas there is a only very slow and weak increase in case of a EO_4 monolayer. As a control experiment, we checked that BAR vesicles do not provide lipids to migrate at the air/water interface if there is no monolayer initially. We did not observe any significant change in surface pressure (less than 1 $mN \cdot m^{-1}$) during more than 80 min after vesicle addition.

In the previous QCM experiments, the adhesion of BAR vesicles was found to be strongly inhibited when the mean molecular area of TAP lipid became larger, particularly when the TAP monolayer was transferred in the liquid expanded phase. Figure 7b shows the surface pressure change in time of the TAP monolayer after injection of BAR vesicles for two initial mean molecular areas of TAP lipid: 0.6 nm^2 corresponding to the LC phase and 1.05 nm^2 corresponding to the LE phase. Both curves show a large increase in surface pressure until reaching a plateau at 38 $mN \cdot m^{-1}$. Interestingly the final value of surface pressure does not depend on the initial mean molecular area of the TAP lipid.

IV. Discussion

A. Vesicle Adhesion to the Deposited Multilayer. The interaction between vesicles and LB multilayers bearing TAP and BAR entities may be expected to occur via formation at the contact area, of "ribbon"-type hydrogen bonded arrays as found in the solid state.[28] To demonstrate the usefulness of the QCM technique for detecting and investigating vesicles adhesion on deposited multilayer, we shall first discuss the significance of the final change in frequency value Δf_{eq} and demonstrate that QCM experiments permit to specify how complementary vesicles

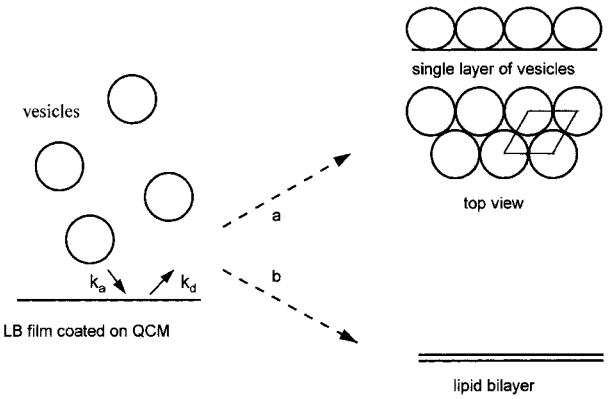

Figure 8. Schematic model of vesicle adhesion.

and surface interact. Considering the attractive interaction between BAR vesicles and a TAP layer, one expects that BAR vesicles adsorb strongly on the TAP surface. Depending on the deformability of vesicles, their adhesion to the surface can lead to two final states (Figure 8): either formation of a single bilayer or of a vesicle layer on the surface, whether vesicles remain intact, rupture, or fuse. Indeed the contact area between a given adsorbed vesicle and a surface is controlled by the balance between bending and adhesion energy.[29,30] When the adhesion energy, and thus the contact area, is increased, the vesicle shape will dramatically change from a sphere to a disk until vesicle rupture or fusion between bound vesicles coming into contact ends up in a single bilayer covering all the surface.[31] In the latter case, the expected frequency change would be around 140 Hz, corresponding to two deposited lipid monolayers.[32] Even if one supposes that there is an additional water layer between such a bilayer and the surface, its thickness would be around 55 nm. Thus one can exclude a strong vesicle adhesion leading to a single bilayer. Now, if one considers vesicles as hard spheres because of their small size, then the best surface coverage by vesicles will be a hexagonal packing as shown in Figure 8. If one assumes that the change in mass corresponds to the lipids coming from all the adhering vesicles, it can be estimated and the change in frequency can be deduced by means of the Sauerbrey equation.[20] For a hexagonal arrangement of vesicles of diameter d, the ratio r_1 of total surface S_{ves} of adhering vesicles per unit cell of the QCM surface S_{quartz} is

$$S_{ves} = \pi d^2 \quad \text{and} \quad S_{quartz} = \frac{\sqrt{3}}{2} d^2$$

$$r_1 = \frac{S_{ves}}{S_{quartz}} = \frac{2\pi}{\sqrt{3}} \approx 3.6 \qquad (4)$$

Interestingly this ratio is independent of vesicle size. The change in mass due to adhering vesicle bilayers is then equivalent to 7.2 TAP monolayers, i.e., about 410 ng if one considers a mean molecular area of 0.4 nm^2. The expected change in frequency, deduced from the frequency shift corresponding to one deposited monolayer, would be around 450 Hz which is not in agreement with the

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(31) Nollert, P.; Kiefer, H.; Jahnig, F. *Biophys. J.* **1995**, *69*, 1447−1455.
(32) Frequency shifts caused by the deposition of 3, 5, or 10 monolayers were measured. The frequency shift $\Delta f(TAP)_n$ due to the deposition of $(TAP)_n$ was found to be proportional to the number n of deposited monolayers: $\Delta f(TAP)_n = n \times 70$ (Hz).

experimental value Δf_{eq}. Second, if one assumes that the adhering vesicles keep their integrity and keep a constant volume and surface, the compartment of an adhering vesicle behaves as an additional mass accumulated on the quartz.[33] Considering the previous model, the ratio r_2 of total vesicle volume V_{ves} per unit cell of quartz surface can be calculated.

$$V_{ves} = \frac{\pi}{6} d^3 \quad \text{and} \quad r_2 = \frac{V_{ves}}{S_{quartz}} = \frac{\pi}{3\sqrt{3}} \approx 0.6d \quad (5)$$

Under such conditions, a frequency change of 1000 Hz corresponds to the adhesion of vesicles of 60 nm diameter d and 2.5 nm monolayer thickness (equivalent to 14.4 monolayers, i.e., 820 ng). This diameter is in very good agreement with the expected size of the SUV prepared by sonication. Another way of viewing the problem is to consider that the vesicle layer, as well as all the water, vibrates with the quartz in a shearing mode. In the latter case, a frequency change of 1000 Hz corresponds to 15 monolayers of 3 nm thickness, i.e., 45 nm, which is also in good agreement with the vesicle size.

In conclusion, the present results support a picture where the BAR vesicles adhere to the supported TAP multilayer and the contact area is reduced enough to maintain the integrity of the adhering vesicle. Moreover the encapsulated water of these vesicles has to be taken into account as an additional mass attached to the quartz. The final frequency change, corresponding to the equilibrium state, indicates that the adhering vesicles keep their integrity at least during the time of the experiment (more than 90 min) and tend to form a single layer covering the entire surface.

B. Kinetics of Vesicle Adhesion to a Complementary Supported LB Multilayer. Another point of interest concerns the kinetics of the observed phenomenon. One can consider vesicles interacting attractively with the surface as hard spheres and interacting repulsively one and all due to the charged amphiphiles and the stabilizing tetraoxyethylenic spacer. By analogy to a Langmuir adsorption model of gas molecules, the rate of mass adsorption onto the surface depends on the ratio σ of the surface covered by vesicles, and the kinetics of vesicle adhesion are described by the following relation:

$$\frac{d\rho}{dt} = k_a(1 - \sigma) - k_d\sigma \quad (6)$$

The first term is the adsorption rate of vesicles, proportional to the adsorption constant k_a[34] and to the ratio $(1-\sigma)$ of the surface noncovered by vesicles, and the second term is the desorption rate of vesicle, proportional to the desorption constant k_d and to the ratio σ of the surface covered by vesicles.

Resolving eq 6 gives

$$\Delta f(t) = \Delta f_{eq}[1 - \exp(-t/\tau)] \quad \text{with} \quad \tau = \frac{1}{k_d + k_a} \quad \text{and}$$

$$\Delta f_{eq} = \Delta f_{max}\frac{k_a}{k_d + k_a} \quad (7)$$

where Δf_{eq} and Δf_{max} are respectively the frequency change at the saturation plateau and the frequency change corresponding to the total coverage of the surface by a layer of vesicles.

It was found that all the curves $\Delta f(t)$ can be well fitted by a monoexponential law corresponding to a Langmuir adsorption model. From the previous relation (6) and the values τ and Δf_{eq} measured, one can deduce that

$$k_a = \frac{\Delta f_{eq}}{\tau \Delta f_{max}} \quad (8)$$

$$k_d = \frac{1}{\tau}\left(1 - \frac{\Delta f_{eq}}{\Delta f_{max}}\right) \quad (9)$$

The adsorption constant k_a depends on several parameters: the vesicle concentration, the diffusion and convection processes, and the coverage ratio. On the other hand, the desorption constant k_d of the vesicles adhering to the quartz surface is directly related to the activation energy of desorption and thus to the affinity of the vesicles for the surface. Assuming that Δf_{max} corresponds to Δf_{eq} in the experiment 1 (see Table 1), one can deduce from eq 9 the k_d value in each experiment. In agreement with a Langmuir adsorption model, when the vesicle affinity for the surface decreases (Δf_{eq} decreases), the global rate increases (τ decreases) and the desorption rate constant k_d increases, so that the equilibrium is reached more rapidly (see Table 1). Moreover the time range of the frequency change, less than 30 min (as observed by optical microscopy), is in agreement with the agregation behavior observed in the case of interacting complementary TAP and BAR giant vesicles.[35]

In conclusion, vesicle adhesion onto a supported multilayer occurs following a reversible process leading to an equilibrium state rather than by a process leading to fusion or rupture of the vesicles onto the surface. The kinetics of vesicle adhesion given by the QCM technique are in good agreement with a model of Langmuir adsorption, and the determination of k_d permits one to quantify the affinity of vesicles for the surface.

C. Selectivity of Vesicle Adhesion to a Complementary Supported Multilayer. Comparison of the previous experiment with that using noncomplementary vesicles (see Figure 2a and Table 1, entries 1−6) gives information about the selectivity of the adhesion process. The data obtained for noncomplementary inert EPC vesicles provide an estimate for the nonspecific adhesion that may be attributed to hydrophobic interaction between the EPC vesicles and the deposited multilayer. These experiments confirm the significance of Δf_{eq} and k_d and thus show clearly the selectivity of the BAR/TAP association compared with TAP/TAP or TAP/EPC. In the case of mixed vesicles containing EPC and the same amounts of BAR and TAP lipids, the affinity of the vesicles for the monolayer disappears completely. This could be due to competitive intravesicular association between TAP and BAR lipids, thus inhibiting the interaction of vesicles with the supported multilayer.

Thus, these QCM experiments show clearly that complementary vesicles and deposited multilayer interact selectively. The comparison between different populations of vesicles offers a simple tool for testing the selectivity of the vesicle/surface interaction.

D. Nature of the Interactions Involved in Vesicle Adhesion. Effects of pH and Ionic Strength on Vesicle Adhesion. In view of the nature of the headgroups of the lipids used, both electrostatic and hydrogen-

(33) We consider that the water density is equal to that of the lipid bilayer: $\rho_{water} \cong \rho_{bilayer}$

(34) k_a depends on the bulk vesicle concentration C, which for this reason is maintained constant in all the experiments.

(35) Results not published.

bonding interactions are expected to contribute to the observed phenomena (Figure 3). The electrostatic contribution results from protonation/deprotonation processes.[26] The vesicle adhesion remains efficient in a large pH range (Figure 4b and Table 1, entries 7−11) and increases significantly when pH decreases as shown by Δf_{eq} and k_d. This result can be explained by the fact that the positive surface charge of TAP vesicles increases considerably from pH close to 7 to lower pH whereas the negative surface charge of BAR vesicles is important until the pH becomes smaller than 4. Thus the electrostatic attraction is expected to be more efficient at pH 4 or 5 than pH 7 or 9.[36] It was also found that vesicle adhesion is weaker in a buffer of 0.1 M ionic strength than in pure water (Figure 4a). At a constant ionic strength of 0.1 M and pH 7, the electrostatic potential created by the protonated groups TAP becomes very weak in comparison with that in pure water (for an ionic strength $<10^{-5}$ M).[37] In the presence of a buffer of 0.1 M ionic strength, the range of electrostatic interaction is very short, the Debye screening length being around 1 nm.[38] These results show that there is an electrostatic contribution to vesicle adhesion. In addition, even if the electrostatic contribution is screened by a high ionic strength, the selective interaction between complementary vesicles and multilayer still efficiently maintains adhesion since the frequency change corresponds to about half coverage of the surface with vesicles.

E. Competition between BAR Molecules and BAR Vesicles. In the competition experiment employing a barbituric acid solution (Figure 5 and Table 1, entry 12), the interaction is very weak in comparison to the case of BAR vesicles in pure water. The supported TAP membrane is initially saturated with barbituric acid molecules since the film was transferred in the presence of barbituric acid in the subphase. Indeed, molecular recognition between monolayers and solutes in the subphase using melamine and barbituric derivatives has been described.[6,39] We can envisage two equilibrium states corresponding to saturation of this surface with either free BAR molecules or BAR vesicles and evaluate the cost to replace BAR molecules by BAR vesicles. Whatever the nature of the interaction between TAP and BAR lipids at the surface, the molecular recognition sites are expected to be more accessible in the case of surface saturation by the free BAR molecules than by the BAR vesicles. Indeed, the BAR lipids movements are restricted within the vesicle bilayer whereas the free BAR molecules can cover more efficiently the surface. Nevertheless, with respect to electrostatic interactions, all the charged lipids of an adhering vesicle contribute to its adhesion because the range of electrostatic interaction at an ionic strength of 10^{-3} M (Debye screening length around 10 nm) is bigger than the vesicle diameter.[36] On the other hand, in the case of hydrogen bonding, the orientational contraints and the short range of such interactions implies that only the TAP and BAR lipids located in the contact area are involved in the BAR/TAP binding. Then, in the latter case, the interactions should be weaker in the case of BAR vesicles than of free BAR molecules whereas in the case of electrostatic interactions, a smaller difference is expected between free BAR molecules and BAR vesicles. As a consequence, the TAP/BAR complex formed at the surface cannot be dissociated by the vesicles so that the BAR molecules inhibite vesicle adhesion.

Table 3. Electrostatic Surface Parameters of Various TAP/EO_4 Mixed Monolayers at pH 7 and in Pure Water[a]

surface ratio in TAP lipids	% of ionized TAP sites	surface electrostatic potential Ψ_o (mV)	surface charge σ_o (C/nm^2)
100	34	4.6	0.86
50	36	2.4	0.46
25	37	1.3	0.23
10	38	0.5	0.10

[a] An ionic strength of 10^{-6} M was taken as the residual salts concentration in pure water. The calculations were done according to a Poisson-Boltzmann model; for details, see ref 26.

F. Effect of Dilution of the Number of Recognition Sites within the Surface. The vesicle adhesion decreases strongly with the ratio of TAP lipids within the supported multilayer (Table 1, entries 13−15) indicating that the interaction depends on the surface density of recognition sites. The decrease of the surface charge and of the surface electrostatic potential (see Table 3) is expected to decrease the affinity of the vesicle for the surface. An additional contribution comes from hydrogen bonding.

At first, the TAP and EO_4 lipids possess the same molecular structure except for the headgroups which are far enough from the alkyl chains so as not to affect markedly the monolayer properties. Indeed ($\pi-A$ isotherms of TAP/EO_4 tend to prove that the two lipids form a solid solution at the air/water interface. Furthermore, DSC measurements of various TAP/EO_4 mixtures in water exhibited no significant changes in the transition temperature or the latent heat (see Table 2). No eutectic point was detected, so that one can exclude a large segregation between the two amphiphiles. Then the TAP/EO_4 mixture may be considered as homogeneous with the TAP lipid distributed within the deposited multilayer.

Because of the high transfer pressure used for preparing supported multilayers, the lipid diffusion is strongly restricted,[40] thus inhibiting a redistribution of TAP lipids within the surface. If one considers the possible *n*/*n* TAP/BAR association to form a "ribbon structure",[27] one would expect BAR lipids to migrate to the contact area when the vesicle is close to the membrane. The constant of lateral diffusion of one single lipid within a bilayer is about 1 $\mu m^2 \cdot s^{-1}$ so that it takes about 80 ms to explore the whole surface of a 50 nm diameter vesicle by diffusion.[35] On the other hand, TAP lipids of the supported membrane cannot diffuse and feed the contact area; then the mean distance between TAP lipids within the supported membrane increases drastically with dilution of TAP with EO_4 so that the *n*/*n* TAP/BAR association by hydrogen bonding cannot be formed. Thus, the number of adhering vesicles decreases dramatically with decreasing density of TAP sites.

This experiment supports a picture according to which the BAR and TAP lipids interact through hydrogen bonding in the contact zone between adhering vesicle and surface and this contribution promotes the vesicle adhesion.

G. Effect of the Tetraoxyethylenic Spacer. As anticipated, vesicle adhesion decreases with the transfer surface pressure which determines the TAP density within

(36) At a 0.1 M ionic strength, the p*K* shift of the ionizable groups is very small compared with that observed in a pure water solution.

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(38) The Debye screening length range was calculated from the following relation: $\kappa^{-1} = 0.308/c^{1/2}$ (nm) where *c* is the 1:1 electrolyte concentration. See ref 37b.

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the supported monolayer. By comparing the mean molecular area of TAP lipids and the corresponding Δf_{eq} and k_d (see Table 1, entries 16−18), one observes a dramatic change in vesicle behavior at the phase transition. In the LE phase, vesicle adhesion is very weak whereas it becomes very efficient in the LC phase. This behavior might be due to the tetraoxyethylenic spacer. Indeed the flexible ethylenoxy chains are tethered so that in the LC phase the TAP sites are well packed and "preoriented" for interaction with BAR. On the other hand, these chains are much more free to move in the LE phase and the increased fluidity of the spacer disorganizes the orientation of TAP headgroup.[41] In contrast to electrostatic interaction, multiple hydrogen bonding is very dependent on the relative molecular orientation, so that the physical state of the supported membrane offers a way for controlling vesicle adhesion.

It has been shown that short PEG-grafted lipids contribute to adhesion by dehydrating the monolayer interface.[42,43] We checked that the tetraethylenic spacer itself does not promote vesicle adhesion. Indeed the vesicle behavior with respect to a EO_4 lipid Langmuir monolayer, used as reference for nonspecific binding (see Figure 7a), permits one to show that the tetraoxyethylenic spacer itself contributes very weakly to the adsorption and to lipid exchange between vesicles and monolayer.

H. Lipid Exchange between Vesicles and a Langmuir Monolayer. Some of the experiments performed concern the interaction between vesicles in water and a Langmuir monolayer. Our aim was to investigate whether if a lipid exchange process takes place between complementary vesicles and a Langmuir monolayer. Previously it was shown that vesicle suspensions can exchange lipids with monolayers at the air/water interface.[44] The mechanism and the kinetics of the lipid exchange have been studied.[45,46] More recently, this approach was used to design functionalized monolayers containing proteins without denaturation that would be caused when an organic solvent is used in spreading.[47] Others studies were carried out in view of quantifying the lipid exchange between a liposome suspension and a Langmuir monolayer both negatively charged in the presence and absence of divalent cations.[48] According to the literature,[43,44] the exchange process can be followed by measuring the surface pressure.

In our experiments, a large surface pressure increase (about 30 $mN \cdot m^{-1}$) is observed on addition of BAR vesicles in the subphase of a complementary Langmuir TAP monolayer (see Figure 7a), indicating that the mean molecular area of a TAP lipid within the monolayer decreases strongly. We checked that a BAR vesicle suspension does not form spontaneously a lipid monolayer at the air/water interface if no monolayer was initially spread so that one can exclude that free lipids migrate from vesicles to the air/water interface.[49] As the total interfacial area is constant in this experiment, such a large increase in surface pressure must be due to lipid migration from the subphase to the interface.

Furthermore, from the QCM experiments, it was shown that vesicles adhere selectively to a complementary surface as it was also observed in the case of complemetary TAP and BAR vesicles.[8] After the addition of complementary vesicles, one can observe a latency time of about 40 min before the increase in surface pressure occurs (see parts a and b of Figure 7). This duration may be related to vesicle diffusion and adhesion onto the monolayer. Contrary to the QCM experiments where lipids movements are inhibited, a lipid exchange process can occur between adhering vesicles and the monolayer in the contact area. The overall lipid migration will take place from adhering vesicles to monolayer until the monolayer and the bilayer reach the same equilibrium surface pressure. Moreover the equilibrium value (38 $mN \cdot m^{-1}$) of the enhanced surface pressure was identical whether the initial mean molecular area was 1.05 or 0.6 nm^2. This value may be imposed by the membrane pressure within the lipidic bilayer of vesicles. Indeed the equilibrium will be reached when the chemical potentials of monolayer and vesicle bilayer, regarded as two phases, have become equal.[44] Considering that the vesicles are in excess, the vesicle bilayers can be regarded as a reservoir with a constant chemical potential which imposes the value of the mean molecular area and thus the surface pressure at the equilibrium. Usually, the internal lateral tension within a vesicle bilayer is on the order of 30 $mN \cdot m^{-1}$,[50] which is close to that measured in our experiment even if the surface pressure of a Langmuir monolayer is not strictly comparable with that of a free bilayer in water.

Contrary to the case of QCM experiments, lipid diffusion is possible within the monolayer, and the complementary vesicles not only adhere to but also exchange lipids with the monolayer following a possible hemifusion process. These preliminary experiments show that it may be possible to follow the hemifusion between complementary vesicles and a Langmuir monolayer by measuring the changes in surface pressure.

V. Conclusion

The present results provide a description of the selective interaction between vesicles and supported lipid layers containing complementary hydrogen-bonding headgroups. The QCM technique used permits to detect vesicle/layer adhesion, to study its selectivity and dependence on various factors and to quantify the process. The effect of several key parameters has been elucidated: ionic strength and pH, concentration of recognition sites, and the state of the supported membrane. They point to an electrostatic contribution to the vesicle adhesion. Moreover all the results support a picture where hydrogen bonding stabilizes the adhesion between oppositely charged vesicles and monolayer by a local arrangement of the complementary lipids in the contact area. The tetraoxyethylenic spacer appears to play a role in the formation of TAP/BAR complexes. Whereas the QCM experiments give information about the adhesion process alone, due to the strong restriction of lipid motion within the supported multilayer, the surface pressure experiments additionnally show that lipid exchange and possibly hemifusion occur between vesicles and the Langmuir monolayer.

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