Dissipation-Enhanced Quartz Crystal Microbalance Studies on the Experimental Parameters Controlling the Formation of Supported Lipid Bilayers

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We report on the investigations of the transformation of spherically closed lipid bilayers to supported lipid bilayers in aqueous media in contact with SiO_2 surfaces. The adsorption kinetics of small unilamellar vesicles composed of dimyristoyl- (DMPC) and dipalmitoylphosphatidylcholine (DPPC) mixtures on SiO_2 surfaces were investigated using a dissipation-enhanced quartz crystal microbalance (QCM-D) as a function of buffer (composition and pH), lipid concentration (0.01−1.0 mg/mL), temperature (15−37 °C), and lipid composition (DMPC and DMPC/DPPC mixtures). The lipid mixtures used here possess a phase transition temperature (T_m) of 24−33 °C, which is close to the ambient temperature or above and thus considerably higher than most other systems studied by QCM-D. With HEPES or Tris·HCl containing sodium chloride (150 mM) and/or calcium chloride (2 mM), intact vesicles adsorb on the surface until a critical density (Θ_c) is reached. At close vesicle contact the transformation from vesicles to supported phospholipid bilayers (SPBs) occurs. In absence of $CaCl_2$, the kinetics of the SPB formation process are slowed, but the passage through Θ_c is still observed. The latter disappears when buffers with low ionic strength were used. SPB formation was studied in a pH range of 3−10, yet the passage through Θ_c is obtained only for pH values above to the physiological pH (7.4−10). With an increasing vesicle concentration, Θ_c is reached after shorter exposure times. At a vesicle concentration of 0.01−1 mg/mL, vesicle fusion on SiO_2 proceeds with the same pathway and accelerates roughly proportionally. In contrast, the pathway of vesicle fusion is strongly influenced by the temperature in the vicinity of T_m. Above and around the T_m, transformation of vesicles to SPB proceeds smoothly, while below, a large number of nonruptured vesicles coexist with SPB. As expected, the physical state of the membrane controls the interaction with both surface and neighboring vesicles.

Introduction

Supported lipid bilayers (SLBs) are synthetic ultrathin organic membranes which serve as model systems for cell membranes[1,2] and as a means to construct surface-based devices.[3−5] Especially, phospholipid bilayers (SPBs) mostly composed of single components,[6,7] but also composed of defined lipid mixtures,[8−12] are interesting candidates for studying elementary processes of bilayer membranes. Natural bilayer membranes, present in all living systems, are even more complex. They provide fundamental functions, like inter- and intracellular communication, for organism survey. Their constituents, phospholipids and proteins, are organized into a complex structure responsible for their properties. Such structural organization has been described as a "fluid mosaic model" via integration of specialized proteins into a liquid-crystalline lipid bilayer.[13] Systems with the full biological complexity can typically not be studied in great detail as it is difficult to vary single parameters. In contrast, simple model systems such as SLBs and especially SPBs have attracted considerable attention for mimicking natural membranes and for studying membrane processes. Since their description by Mueller et al. in the 1960s,[14] supported phospholipid bilayers have received increasing practical and scientific interest due to their application potential in life science (biosensors, library screening) and natural properties (environment).[1] They are typically formed on silica surfaces by a single two-dimensional phospholipid bilayer either free or connected to the surface (tethered). The bilayers are separated from the solid support by an ultrathin film of water or polymer. Either this nanometer-thick water layer (1−2 nm) or ultrathin soft polymer cushion, embedded between the solid support and the lower leaflet of the bilayer, maintains the thermodynamic and structural properties of the free bilayers.[15] Due to their well-defined geometry, they are convenient to study with sensitive techniques for surface analysis including surface plasmon resonance (SPR),[16] quartz crystal microbalance (QCM),[17,18] impedance spectroscopy,[19] attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR),[20] scanning probe microscopy (in situ AFM),[21,22] and fluorescence recovery after photobleaching (FRAP).[2,8]

Early on, SPB formation was often performed by sequential deposition of two lipid monolayers onto a suitable solid support using the Langmuir−Blodgett technique (LB).[23] However, more recently, McConnell et al. described planar lipid bilayer creation by spontaneous vesicle spreading.[24] This method is more convenient and nowadays commonly used for SPB preparation

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on hydrophilic and hydrophobic surfaces.[17] Another advantage of this method is the possible incorporation of functionalized molecules (proteins, ...) to the membrane for precise applications.[1,2,25] Today, intense research is focused on developing an understanding and controlling of both the kinetics of the SPB formation process and the properties of the SPBs formed. Recent development of surface analytical tools, such as atomic force microscopy (AFM) and dissipation-enhanced quartz crystal microbalance (QCM-D), has opened the way for getting answers to several questions. Kasemo et al. have recently studied the influence of several experimental parameters (temperature, size of vesicles, surface nature, and osmotic pressure) on SPB formation using the QCM-D technique.[9,26] In the same way, AFM has allowed both in situ observation of SPB formation via intermediate nanoscopic patches[10,11,21,27] and topographical studies on supported membranes.[6,22] Recent work of Brisson et al. carried out on SiO_2 and mica substrates has allowed identification and characterization of a multitude of processes that take place from the adsorption of vesicles to complete bilayer formation using combined QCM-D and AFM techniques.[10,28] Furthermore, fluorescent-labeled vesicles have been used recently to study the pathway of SPB formation.[29] Despite all these efforts, several questions on experimental parameters (surface, lipid composition, temperature, ...) influencing the SPB process are still open.

At the same time, studies of the predominant membrane constituent, the phospholipids, have been performed for several decades to arrive at a more quantitative understanding of membrane behavior and lipid interactions. A cell membrane is composed of a complex mixture of different kinds of lipids. Therefore, theoretical and experimental studies on the mixing behavior of different lipids in hydrated liquid-crystalline bilayers have been carried out.[30−32] Lipid dispersions have been studied as a function of temperature by various methods. The resulting phase diagrams show a main transition temperature (T_m), which corresponds to the gel to liquid transition. This transition temperature depends on the nature of lipids such as chain length, type of acyl chain, charge, and headgroup, parameters which are also responsible for miscibility or phase separation within a mixture. Mixing and phase separation have been observed for a large number of lipid mixtures, for example, by differential scanning calorimetry (DSC) and atomic force microscopy (AFM).[33,34]

In the literature, it has been shown that vesicle spreading is temperature dependent on SiO_2[9] and glass[35] surfaces, while on mica the SPB formation process seems to be differently induced.[22] However, several recent studies on various supports (SiO_2, mica, positively and negatively charged surfaces) using mixtures of zwitterionic, negatively and positively charged lipids have shown the determining role of electrostatic interactions on lipid deposition.[10,12,18,28,36] SPB formation has been mainly observed above the melting temperature of the corresponding lipid.[6] Experimental constraints frequently impose to work with lipids and lipid mixtures that are in the liquid state at ambient temperature (25 °C).

A powerful analytical tool for in situ studies on the process of vesicle adsorption/fusion and the formation of complete bilayers is dissipation-enhanced quartz crystal microbalance (QCM-D). It allows observation of two parameters simultaneously: the adsorbed mass (as a frequency shift) and the surface viscoelastic behavior (as a change in dissipation) as a function of both time and temperature. The temperature can be varied between 15 and 45 °C with a precision of 0.05 °C. The resolution in time is about 0.5 s with a baseline stability that allows monitoring an experiment over 36 h or more.

Here, we study the influence of five physical parameters, namely, buffer composition and pH, vesicle concentration, temperature, and lipid composition, on the SPB formation of DMPC/DPPC lipid mixtures using the QCM-D technique. These results have allowed identification of which parameter plays a role in the kinetics and/or mechanism in the process of bilayer formation. To our knowledge, the present work is the first systematic study on SPB formation close to the vesicle phase transition temperature (T_m). The use of well-defined binary lipid mixtures allows better control of the process of vesicle adsorption and membrane fusion and extension of the understanding of the process as known from single-component systems.

Material and Methods

A. Materials. 1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine (99%, DMPC, melting temperature of 24 °C) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (99%, DPPC, melting temperature of 41 °C) were purchased from Sigma (St. Louis, MO) and Avanti Polar Lipids (Alabaster, AL), respectively. For buffer preparation, 4-(2-hydroxyethyl)piperazine-1-ethane sulfonic acid (HEPES, 99.5%), tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl, 99%), anhydrous calcium chloride (97%), and sodium chloride (99.5%) were purchased from Fluka (Buchs, Switzerland), Acros organics (Geel, Belgium), Lancaster (Mülheim, Germany), and Merk (Darmstadt, Germany), respectively. All chemicals were used without further purification. Water used throughout this study was purified with a milli-Q water purification system (Millipore, Molsheim, France). For most experiments the buffer composition was as follows: 10 mM of Tris·HCl or 20 mM of HEPES, 150 mM of NaCl, and 2 mM of $CaCl_2$ (B3 or B7). The seven following buffers were employed for specific experiments: (B1) 20 mM of HEPES; (B2) 20 mM of HEPES, 150 mM of NaCl; (B3) 20 mM of HEPES, 150 mM of NaCl, 2 mM of $CaCl_2$; (B4) 10 mM of Tris·HCl; (B5) 10 mM of Tris·HCl, 2 mM of $CaCl_2$; (B6) 10 mM of Tris·HCl, 150 mM of NaCl; (B7) 10 mM of Tris·HCl, 150 mM of NaCl, 2 mM of $CaCl_2$. In all cases, the buffer pH was adjusted to 7.4 using 0.5 M NaOH except for the pH study where pH values of buffer (B7) were 3, 5, 6, 7, 7.4, 8, 9, 10, and 12. Low pH values were adjusted using 0.6 M HCl.

Chemicals used for cleaning were chloroform (99%, Merk, Germany), ethanol (99%, Carlo Erba reagent, France), hellmanex (Hellma, France), and nitrogen (alphagaz N_2 1, Air liquide, France).

B. Preparation of Vesicles. Lipid mixtures were obtained by mixing the appropriate volumes of lipid solutions in chloroform. Then the organic solvent was removed in a nitrogen flow. The resulting lipid film was dried for one night under vacuum. To form mulilamellar large vesicles (MLVs), the lipids were resuspended in the respective buffer at a concentration of 2 mg/mL.

Small unilamellar vesicles (SUVs) were produced from MLV suspensions by sonication to clarity (12 min, in continuous mode) at room temperature with a tip sonicator (Branson Sonifier B15 Cell Disrupter, Danbury, Ct., USA). Titanium particles were removed by centrifugation (10 min at 5000 rpm) (Biofuge pico, Heraeus, Kendro, France). All the studies were performed after diluting the SUV suspensions to a concentration of 0.05 mg/mL except for the concentration study where it was 0.01, 0.025, 0.05, 0.1, 0.3, and 1 mg/mL. In all cases, the buffer was the same inside and outside the vesicles.

C. Determination of Size Distribution. The size of the lipid vesicles formed was measured at 25 °C by dynamic light scattering (Zeta Sizer 3000 HS, Malvern Instruments, Ltd., U.K.) at a scattering angle of 90°. Only SUV dispersions with a narrow size distribution and a mean diameter of approximately 50 nm were used.

D. Determination of Phase Transition Temperatures. DSC measurements were performed on MLV suspensions of 2 mg/mL using a differential scanning calorimeter (MicroDSC III, Setaram, Caluire, France). Heating rates of 0.25 and 0.5 °C/min were used in temperature intervals from 10 to 60 °C. Measured T_m values for DMPC/DPPC mixtures with the composition 70/30 and 50/50 were 29 and 33 °C (peak center), respectively, in agreement with the literature.[30]

E. Dissipation-Enhanced Quartz Crystal Microbalance (QCM-D) Experiments. All experiments were performed using a QCM-D D 300 instrument equipped with a QAFC 302 chamber and SiO_2-coated quartz crystals (Q-Sense AB, Göteborg, Sweden). It has been described in detail by Rodahl et al. in 1995.[37] In the fluid cell thermally insulated, the crystal is mounted horizontally and the exchange of the solutions is achieved perpendicularly to the crystal surface. The fluid enters from a temperature-controlled loop before being introduced into the measurement cell. A Peltier element controls the temperature with an accuracy of ±0.05 °C. AT-cut quartz crystals (14 mm in diameter) with a fundamental resonance frequency of 5 MHz were used in this study. The piezoelectric crystal is excited at its fundamental resonance frequency, and the observation takes place at the third, fifth, and seventh overtones (denoted n where $n = 3$, 5, or 7) corresponding to 15, 25, and 35 MHz, respectively. The relation between the adsorbed mass and the corresponding frequency change is described by the Sauerbrey equation (*A*) where C (=17.7 ng/(cm^2/Hz) at $f = 5$ MHz) is the mass-sensitivity constant and n (=1, 3, 5, or 7) is the overtone number.[38]

$$\Delta m = -\frac{C}{n}\Delta f \qquad \text{(A)}$$

Equation (A) is valid only for rigid films. In addition, this instrument allows simultaneously measurement of an energy dissipation factor, D, which gives information about the viscoelastic properties of the adsorbed film. Data acquisition was performed with Q-Soft 301 (version 1.6.4) (Q-Sense AB) and data interpretation with Q-Tools (version 2.04) (Q-Sense AB). All data shown for the frequency shifts are normalized to the response of a 5 MHz crystal.

Sample Preparation for QCM-D. Before each experiment, all crystals are UV/ozone treated for 15 min in a home-built UV/ozone chamber and rinsed alternatively with Milli-Q water and ethanol. Then, a second UV/ozone treatment is applied for 30 min, yielding SiO_2 surfaces that are highly hydrophilic and that lead to very reproducible results. In all QCM-D experiments, the cell is initially filled with buffer and rinsed several times until a baseline is established at the desired temperature. When the baseline is stable, the buffer is exchanged for a solution containing vesicles and data were collected as a function of time by recording the changes in f and D. All QCM-D experiments were repeated several times for each experimental parameter. Only one original trace is shown in the paper for reasons of clarity. For given experimental conditions, total frequency shift and total dissipation shift were reproducible to within ±1 Hz and $\pm 0.1 \times 10^{-6}$, respectively, for measurements performed at temperatures higher than 20 °C. For experiments carried out at 15 °C, the same behavior was observed several times but with slightly higher difference in frequency and in dissipation. In addition, the time needed to form a complete bilayer shows some variability (±0−30 s).

Results

1. Investigations Using Vesicles Composed of Single Lipids. 1.1. SPB Formation as a Function of Buffer Composition. There are three major factors that are expected to have an influence on vesicle adsorption and membrane fusion: (1) the nature of the buffer salts, (2) the ionic strength, and (3) the pH value. All of these parameters are able to influence the vesicle−surface interactions and the vesicle−vesicle interactions controlling the process of bilayer formation.[9] There are already reports in the literature investigating the SPB formation in different kind of aqueous buffers.[8,9,22]

Few studies on buffer composition have shown the influence of the buffer constituents on the SPB process. Recently, the effect of the ionic strength on SPB formation has been studied by FRAP[8] and QCM-D experiments.[9] The latter, performed in a range of 115−300 mM of NaCl, show SPB formation; however, the exposure time and the amplitude of the Δf and ΔD peaks decrease when the osmotic pressure increases. Brisson et al. have studied on mica and silica the influence of calcium ions on the bilayer formation using mixtures of zwitterionic, negatively and positively charged lipids.[10,28] They have demonstrated that the effect of calcium on the kinetics of bilayer formation is more pronounced on mica than on silica. In the present paper, the QCM-D technique was used to study buffer composition on SiO_2 and to evaluate its influence on the formation of SPBs. All experiments were performed with DMPC vesicles at 0.05 mg/mL and at 33 °C, 8 °C above the T_m.

The time course of Δf and ΔD upon contact of a SiO_2 surface with DMPC suspensions as a function of buffer composition is represented in Figure 1.

All buffers used for this study had the pH adjusted at 7.4. With typical buffers such as **B3** and **B7** containing the buffer salts NaCl and $CaCl_2$, SPB formation, as described by Kasemo[17] for lipid vesicles having a low T_m (<5 °C) at room temperature, consists of an adsorption step until a critical density of adsorbed intact vesicles (Θ_c) on surface is realized followed by the vesicle rupture. This critical density of vesicles related to the minimum in frequency and the maximum in dissipation.

A few seconds after injection of the vesicle solution, the frequency decreases (mass uptake) and simultaneously the dissipation increases (more viscous film) until Θ_c is reached. Following the pathway of SPB formation described by Kasemo,[17] the vesicle rupture starts at this point and a minimum in Δf (maximum mass uptake) and a maximum in ΔD are observed. In each case, a minimum Δf value around 30−50 Hz suggests that complete coverage of the surface is not needed to induce the vesicle rupture. Then, Δf increases (mass loss) and ΔD decreases (more rigid film) following the vesicle rupture. Finally, frequency and dissipation curves stabilize at typical values (Δf_{stab} and ΔD_{stab}, respectively) for the formation of a homogeneous lipid bilayer, $\Delta f_{stab} \approx 26$ Hz and $\Delta D_{stab} \approx 0.3 \times 10^{-6}$. No significant change of the baseline could be observed upon rinsing.

What is the role of sodium and calcium with respect to SPB formation? To answer this question, the buffer composition was varied.

Using buffers **B1** and **B4** composed only of 20 mM HEPES and 10 mM Tris·HCl, respectively, SPB formation seems to take place but through a different pathway and slower kinetics. The observed QCM-D responses indicate a fast adsorption−

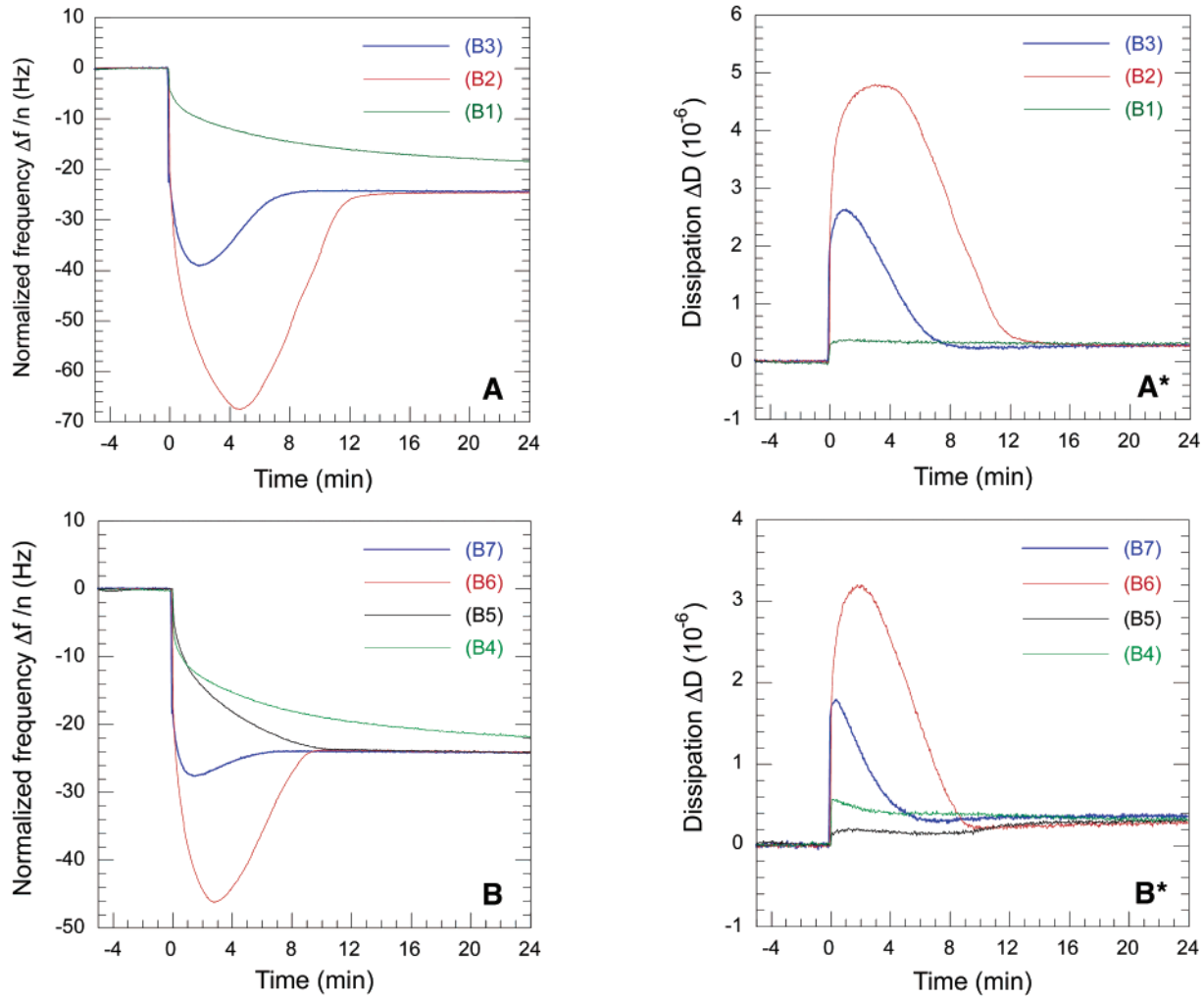


Figure 1. Influence of different buffer compositions on SPB formation on SiO_2 displayed as $\Delta f(t)$ (left) and $\Delta D(t)$ (right) for DMPC (0.05 mg/mL, pH = 7.4, 33 °C): HEPES buffers (A and A*) (**B1**) 20 mM of HEPES; (**B2**) 20 mM of HEPES, 150 mM of NaCl; (**B3**) 20 mM of HEPES, 150 mM of NaCl, 2 mM of $CaCl_2$; Tris·HCl buffers (B and B*) (**B4**) 10 mM of Tris·HCl; (**B5**) 10 mM of Tris·HCl, 2 mM of $CaCl_2$; (**B6**) 10 mM of Tris·HCl, 150 mM of NaCl; (**B7**) 10 mM of Tris·HCl, 150 mM of NaCl, 2 mM of $CaCl_2$. QCM-D results show the importance of each constituent of a buffer with respect to the kinetics and the pathway of SPB formation. In all cases, supported membranes are formed. However, a critical density of vesicles on the surface (Θ_c) is only observed in the presence of sodium chloride. Thus, two pathways of SPB formation can be distinguished: in the presence and in the absence of NaCl. On the other hand, calcium chloride accelerates the kinetics of SPB formation without modifying the existing pathway.

rupture process of the vesicles without passing through Θ_c. However, standard values for Δf_{stab} and ΔD_{stab} are obtained after about 100 min. No significant further change of the baseline could be observed upon rinsing.

With buffers **B2** and **B6** containing 150 mM of sodium chloride, the formation of SPB follows the pathway where a critical density of vesicles is needed to induce their rupture. The electrostatic screening seems to modify the vesicle properties and their behavior toward the surface explaining the mechanistic differences. The comparison with buffer **B3** and **B7**, described previously, shows a similar behavior except that the kinetics of the process is accelerated by the presence of calcium. However, the experiment with buffer **B5** shows that the presence of calcium has only a kinetic effect and no influence on the formation pathway.

Due to solubility problems in HEPES buffer for some lipids, buffers based on Tris·HCl were used for all subsequent experiments.

From the buffer study, we have demonstrated the mechanistic and kinetic importance of the sodium chloride and the calcium chloride in the SPB formation via a critical density of vesicles on surface (Θ_c). On the basis of these results, we also defined buffer **B7** as a standard buffer which has been used for all other experiments.

1.2. Dependence of SPB Formation on Buffer pH. Boxer et al. have described vesicle spreading on glass as a function of pH and ionic strength.[8] Different behaviors of the vesicles on the surface have been reported depending on the charge of the phospholipid headgroup (neutral, anionic and cationic).

For zwitterionic lipids, SPB formation could be observed by FRAP experiments over a pH range of 2.5−12.3. Here, the investigation of the influence of the pH on SPB formation, especially on the kinetic and mechanistic aspects, was carried out on SiO_2 using the QCM-D technique within a pH range of 3−12. All experiments were done for DMPC vesicles at 0.05 mg/mL and at 33 °C, 8 °C above the T_m.

The time course of Δf and ΔD upon exposure of SiO_2 surface to DMPC vesicles as a function of pH is depicted in the parts A and A* of Figure 2. For a pH between 3 and 7, SPB formation is obtained without observation of the critical coverage point. In the case of a pH value between 7.4 and 10, typical curves of SPB formation, where Θ_c could be visualized, were obtained. For pH = 12, no vesicle adsorption and no supported membrane formation could be detected due to dissolution of the SiO_2 layer

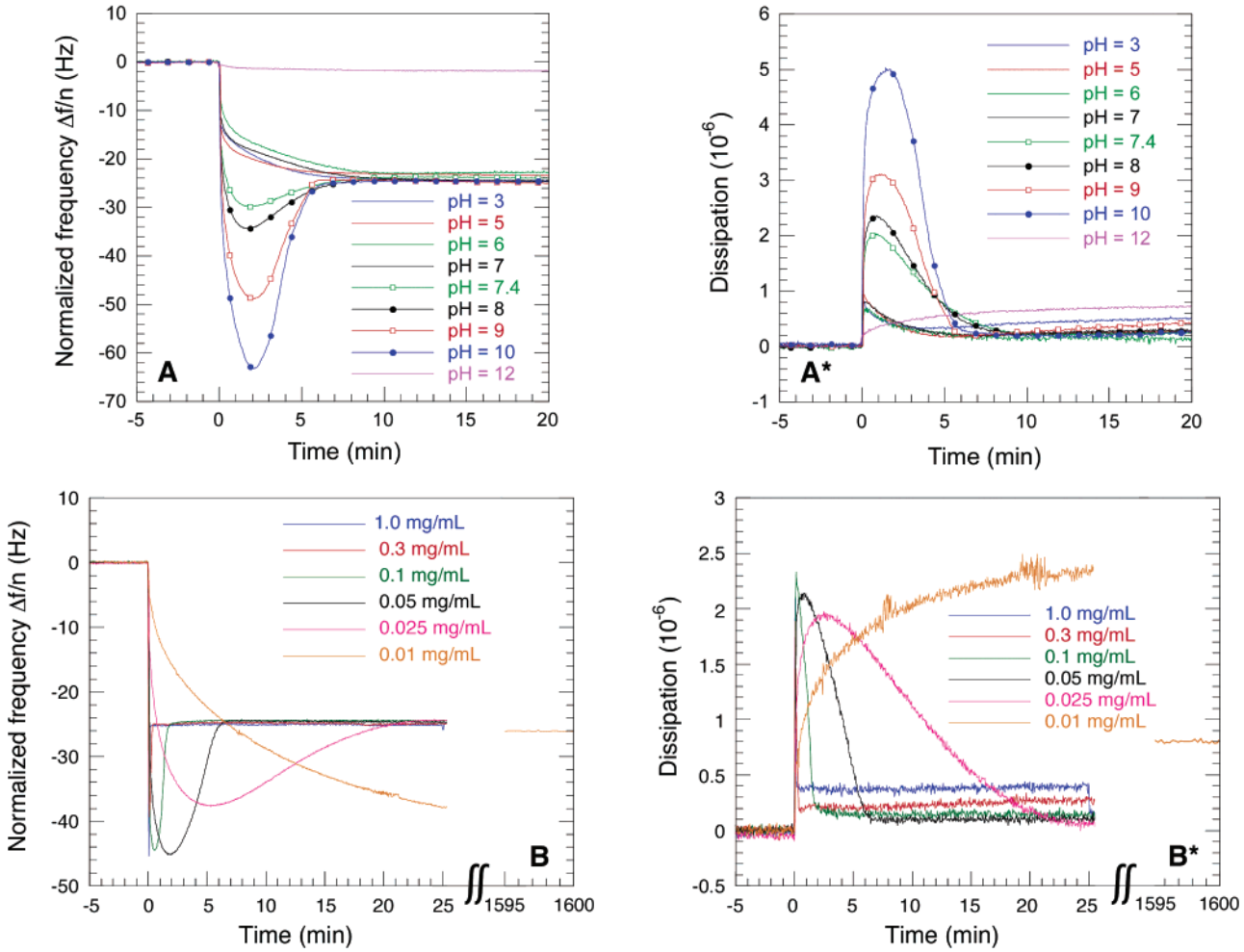


Figure 2. (A and A*). $\Delta f(t)$ and $\Delta D(t)$ QCM-D responses during DMPC (0.05 mg/mL, 33 °C) vesicle adsorption on the SiO_2 surface as a function of the pH of the buffer: 3, 5, 6, 7, 7.4, 8, 9, 10, and 12. SPB formation is observed in the tested pH range except for high pH where the SiO_2 surface is dissolved. However, the SPB formation via a critical density of vesicle (Θ_c) is observed only for pH values between 7.4 and 10. (B and B*). Influence of vesicle concentration (0.01, 0.025, 0.05, 0.1, 0.3, and 1 mg/mL) on SPB formation diplayed as $\Delta f(t)$ (B) and $\Delta D(t)$ (B*) for DMPC (pH = 7.4, 33 °C). QCM-D data show that the kinetics of SPB formation is concentration dependent. The lower the concentration, the higher the time needed for a complete coverage of the surface. However, the pathway of SPB formation is independent of the concentration. The adsorption of the vesicles takes place before vesicle rupture. A shift of Θ_c to higher exposure time is observed upon decreasing the concentration of vesicles.

on the surface of the gold electrode of the quartz crystal.[39] In conclusion, we have shown for the first time that the pH of the buffer controls also the pathway of bilayer formation similar to sodium chloride. To visualize the vesicle rupture and keep a physiological environment, a pH of 7.4 was chosen as standard for all subsequent experiments.

1.3. Influence of Vesicle Concentration on the SPB Formation. From the results shown in the two previous paragraphs, it is clear that the transformation from vesicles to SPBs is influenced by the buffer composition and pH. Another obvious factor that can influence the SPB formation is the vesicle concentration. By increasing or decreasing the vesicle concentration, it is possible to control the number of adsorbed vesicles on the surface and thus the kinetics of the process. Recently, Kasemo et al. have reported SPB formation as a function of concentration for small unilamellar vesicles of eggPC.[40] They have shown that the peak height did not vary with the adsorption rate. Thus, they suggest that vesicle rupture begins at a critical density of vesicles on the surface. In the present paper, the dependence study of vesicle concentration on SPB creation was investigated for pure DMPC at 33 °C, 8 °C above its T_m.

Parts B and B* of Figure 2 show one representative set of QCM-D measurements in which vesicle adsorption and supported bilayer formation on SiO_2 are realized as a function of vesicle concentration. The kinetics of adsorption are displayed as Δf and ΔD versus time at six different concentrations. By increase of vesicle concentration, the minimum in Δf and the maximum in ΔD are reached faster. While a concentration equal or higher to 0.3 mg/mL induces SPB formation quasi instantaneously, the kinetics becomes very slow with concentrations lower than 0.025 mg/mL. Between those values, typical kinetics are observed. In each case, frequency and dissipation suggest that adsorption of vesicles takes place before vesicle rupture occurs. For all concentrations, QCM-D curves stabilized around 25 Hz for Δf and 0 to 0.5×10^{-6} for ΔD.

This study shows that the kinetics of SPB formation are dependent on the concentration of the vesicles and that the pathway of SPB formation is concentration independent. By increase of the concentration, the kinetics of SPB formation is accelerated but the pathway stays the same.

A standard concentration of 0.05 mg/mL was chosen for all subsequent experiments in order to follow each step of the SPB formation on a reasonable time scale (around 10 min).

2. Investigations Using Vesicles Composed of Binary Lipid Mixtures. 2.1. Influence of Temperature and Lipid Composition on SPB Formation. Until now, only vesicles and bilayers composed of pure DMPC were studied as a function

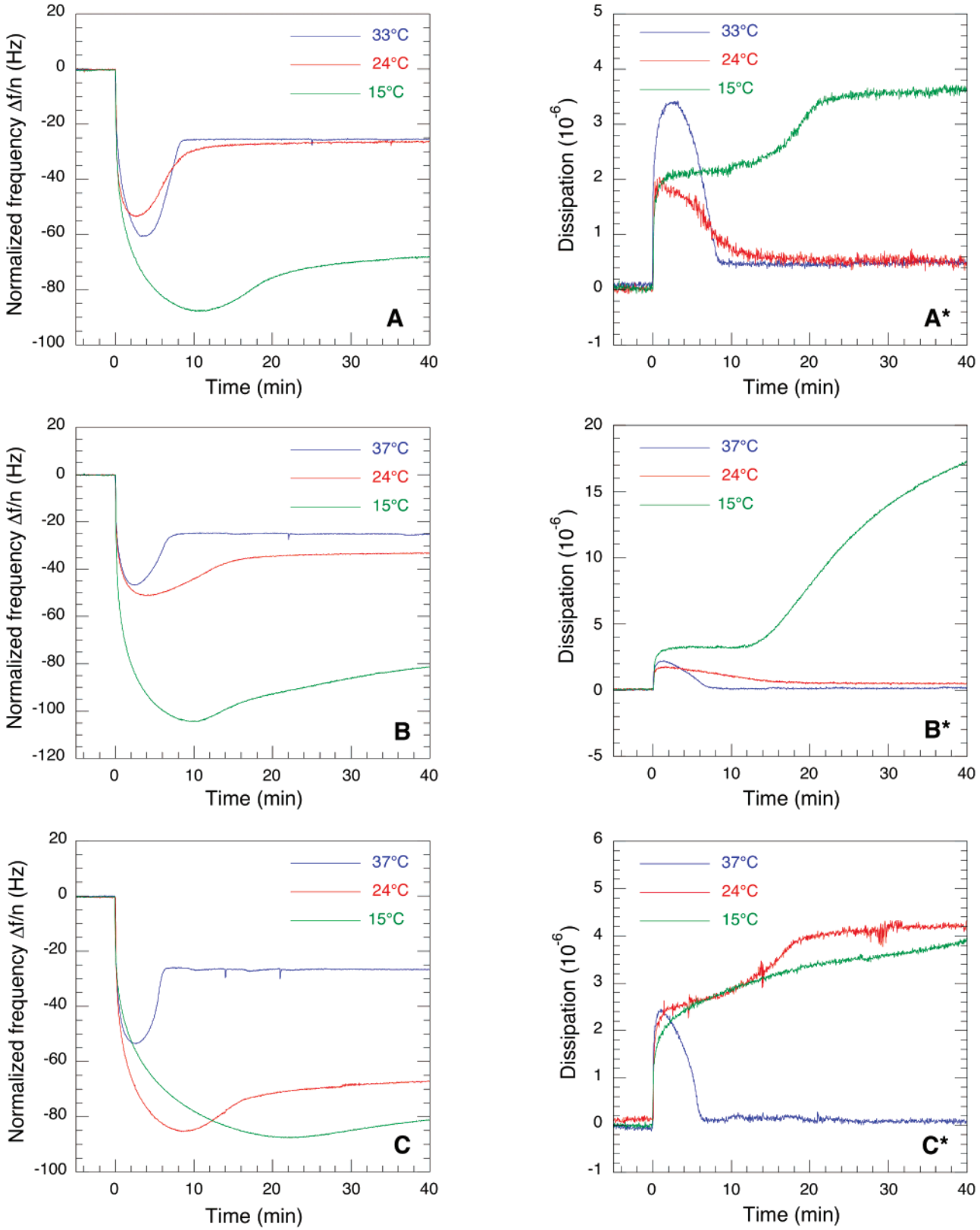


Figure 3. Influence of the temperature on SPB formation on SiO_2 displayed as $\Delta f(t)$ (left) and $\Delta D(t)$ (right) for pure DMPC (A and A*), DMPC/DPPC 70/30 (B and B*), and DMPC/DPPC 50/50 (C and C*). The results show that lipid composition affects the SPB formation depending on the temperature. In all cases, pH and lipid concentration were 7.4 and 0.05 mg/mL, respectively. Above the T_m (37 or 33 °C for DMPC), complete bilayers are observed. At 24 °C, DMPC shows the same fusion behavior but with slower kinetics, while for DMPC/DPPC mixtures low-frequency values are observed. At 15 °C, low-frequency values are observed in all cases.

of different parameters at 33 °C, 8 °C above its T_m. Now, we extend the study to mixtures of different lipids.

Especially, the dependence of lipid composition and T_m value on SPB formation will be investigated. The group of Kasemo has recently shown that the kinetics of SPB formation are temperature dependent.[9,26] On SiO_2 surfaces, the shift of the critical density of vesicles on the surface (Θ_c) to lower exposure time is observed with increasing temperature. To better under-

stand the pathway on SPB formation, we studied the temperature dependence of different DMPC/DPPC mixtures (mixtures 50/50, 70/30, and 100/0) on SPB formation as a function of their T_m values (33, 29, and 24 °C respectively).

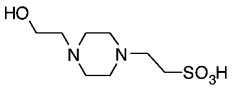
For each mixture, QCM-D investigations were performed above the T_m (37 °C except for pure DMPC where it was 33 °C), around the T_m (24 °C), and below the T_m (15 °C). This way, we have studied the influence of the membrane structure (gel, ripple, or fluid phase) of the vesicles on SPB formation. A small fraction of these data have already been discussed in a different context, namely, to support AFM studies on the formation of DMPC/DPPC mixtures close to their T_m (24 °C).[11]

Figure 3 shows the time course of Δf and ΔD upon exposure of SiO_2 surface to vesicle solutions of (a) pure DMPC, (b) DMPC/DPPC 70/30, and (c) DMPC/DPPC 50/50 at 37 °C (33 °C for DMPC), 24 °C, and 15 °C. Above the T_m, in the fluid state, the observed kinetics are in all cases qualitatively similar to those described by Kasemo for SPB formation.[17]

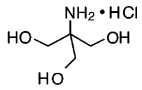
Close to the T_m (24 °C), the time course of Δf and ΔD is dependent on the T_m value of the corresponding lipid mixture. For DMPC, 24 °C is the T_m value while for DMPC/DPPC mixtures, it is slightly lower (29 °C for 70/30 and 33 °C for 50/50). The frequency and dissipation deviation from the typical curves, described previously, becomes higher by increasing the percentage of DPPC in the mixture. Figure 3 shows also that the critical coverage of the surface is reached later and at a lower frequency with higher amounts of DPPC. These results suggest that more vesicles adsorb to the surface before vesicle rupture starts. Moreover, due to a lower thermal activation, the observed kinetics in the rupture process are slower for DMPC/DPPC mixtures, while for pure DMPC kinetics is slightly affected. Hence, a stable baseline in both frequency and dissipation is reached only after longer times for mixtures as compared to pure DMPC. Dissipation curves of the 50/50 lipid mixture show a two-step formation (stable at $\Delta D \approx (4-6) \times 10^{-6}$) while the others exhibit a one-step D response and a lower shift ($\Delta D \approx (0-0.5) \times 10^{-6}$). In the first case, the critical density of vesicles (Θ_c) as seen as a minimum for Δf corresponds to the second increase in the two-step dissipation curves. These two behaviors correspond to the following kinetics: (i) For DMPC/DPPC mixtures, intact vesicles and SPB regions are present on SiO_2 surface. The percentage of intact vesicles on surface increases with an increasing proportion of DPPC in the mixture. Dissipation data suggest the formation of a rigid film for the 70/30 mixture (low D value) while a viscous film is formed for the 50/50 lipid mixture (high D value). (ii) For DMPC, a complete SPB is obtained. Somewhat more time is needed for the SPB formation as compared to the experiment carried out at 33 °C.

At 15 °C, below the T_m, the time course of Δf and ΔD is still dependent on the T_m value. However, a similar Δf and ΔD behavior is observed in all cases: incomplete SPB formation. Figure 3 indicates that the following takes place as a function of frequency: (i) The time required to reach Θ_c (t_{Θ_c}) increases with an increasing percentage of DPPC. (ii) The observed kinetics during vesicle rupture decrease drastically with an increasing fraction of DPPC in the mixture. (iii) For temperatures below T_m, a comparable minimum value for Δf (90−100 Hz) in each case suggests a majority of intact vesicles adsorbed on the surface. (iv) A Δf_{stab} around 60 Hz is recorded which corresponds to coexistence of intact vesicles and SPB regions on the surface. (v) From the Δf_{stab} value, we can concluded that more intact vesicles are present on the surface at 15 °C than at 24 °C. In dissipation, (i) for temperatures below the T_m, a two-step increase is observed for each sample due to simultaneous adsorption of vesicles and vesicle rupture. (ii) A superposition of Δf and ΔD curves shows that this two-step behavior is the consequence of the rupture of some vesicles around Θ_c leading to a dissipation stabilization. Then, vesicle rupture process decreases leading to dissipation increase. (iii) High ΔD_{stab} values are obtained after stabilization indicating a viscous film due to the presence of intact vesicles on the surface. (iv) The factor 3 between the ΔD_{stab} values of DMPC and the mixtures is probably due to a surface reorganization because no difference in mass uptake is observed.

CHART 1: Chemical Structures of HEPES (1) and Tris·HCl (2)



1



2

We show here that the major role of temperature at which SPB formation takes place is important also for the lipid mixtures. As a function of temperature, supported lipid bilayers or intact vesicles surrounded by SPB are observed.

Our studies have shown the effect of several parameters on the kinetics and the pathway of bilayer formation. By tuning these parameters, it is possible to control the process of bilayer formation. Thus, from the results presented here, we can distinguish two mechanistic pathways for SPB formation as a function of the experimental parameters.

The classical one, described by Kasemo,[9,17,41] is called pathway 1. It is based on an adsorption−rupture process including a passage through a critical vesicle density on the surface (Θ_c).

The second one, described by Kasemo[41] and Brisson,[10] is named pathway 2. It is based on a "direct" rupture process upon surface contact where no passage through Θ_c is observed.

Discussion

The results described previously will be discussed here and combined with data from literature in order to obtain a clearer picture for the understanding of the transformation from vesicles to SPBs. In the present paper, we studied a lipid and lipid mixtures possessing a high T_m. The influence of the physical nature (fluid or gel state) of lipids in the mixture on the SPB formation was investigated using QCM-D. In addition, the influence of a larger set of experimental parameters, including buffer, vesicle concentration, and temperature, on the transformation of vesicles to SPB was studied.

1.1. The Dependence of Buffer Composition. All experiments were done with DMPC vesicles in their fluid state to prevent any thermal effects. The results from the experiments with buffers **B1**−**B7** demonstrate the importance of each buffer constituent in the SPB formation process (Figure 1). Without salts (**B1** and **B4**), SPB formation on SiO_2 surface is rather slow as shown by the time dependence of Δf; over 100 min is needed to form a complete bilayer. The kinetics are faster for Tris·HCl than for HEPES buffer, due to the difference in their chemical structure (Chart 1), as described by Rapuano et al.[42] The absence of a minimum in Δf and a small value of ΔD indicate that vesicles seem to break after a short interaction time with the surface (pathway 2). Thus, no critical density of vesicles on SiO_2 (Θ_c) was observed for such buffers. These observations suggest that in the absence of salts, strong vesicle−surface interactions take place and favor a fast rupture process. Indeed, these interactions (van der Waals and electrostatic) between the

surface and the lipids, favoring a greater contact area, destabilize the vesicles and lead to their rupture.[43] However, the slow rate of SPB formation could result from an electrostatic repulsion between the vesicles in solutions and the individual SPB patches. Similar results have been reported recently on SiO_2 by Brisson et al. using positively charges vesicles in the presence and in absence of calcium.[10] In the present work, we can speculate that the positive charge of DMPC (ammonium moiety), located on the outer part of the membrane, acts in a similar way.

The addition of sodium chloride in the buffer composition (**B2** and **B6**) modifies clearly the pathway of SPB formation (pathway 1 is observed). With increase of the ionic strength, the properties of the vesicle membrane and of the surface change due to charge shielding by electrostatic interactions.[43] In this way, a lower adhesion strength between the surface and the lipids stabilizes the vesicles on surface. In these conditions, the adsorption of vesicles takes place until Θ_c is reached, followed by their rupture and the SPB formation. These classical QCM-D curves suggest that the transition of intact vesicles to SPB seems to be caused by a combination of vesicle−surface and vesicle−vesicle interaction.[9] Kasemo et al. have shown that an osmotic stress between the inside and the outside of the vesicles tends to decrease the time needed to reach Θ_c (t_{Θ_c}) and the barrier rupture.[9] Here, without salt gradient applied to the vesicle membrane, a similar behavior is observed by simply adding NaCl to the buffer. The complete bilayer is formed at $t = 15$ and 11 min (buffers **B2** and **B6**, respectively) instead of a time scale >100 min for buffers **B1** and **B4**. As previously, the kinetics in Tris·HCl buffer are faster than those with HEPES. The presence of sodium chloride favors the adsorption of vesicles and their stability on SiO_2 surface until Θ_c where vesicle−vesicle interaction induces the rupture.

Finally, t_{Θ_c} can be decreased by adding calcium chloride (buffers **B3** and **B7**). Divalent ions, such as Ca^{2+} and Mg^{2+}, are known to accelerate membrane fusion processes in general by interacting with the phosphate group of the lipids and the negatively charged surface like SiO_2 and mica.[10,28,44,45] As a consequence, the adhesion strength between the lipids and the support increases. Thus, the adsorption of fewer vesicles on the surface is needed (onset of fusion at smaller values of Δf) to induce their rupture and the SPB process goes to completion in a shorter time scale. However, the overall pathway (pathway 1) of SPB formation is identical to buffers **B2** and **B6**. Again, faster kinetics are observed for Tris·HCl as compared to HEPES.

In addition, the experiment performed with buffer **B5** confirms that the calcium has only accelerated SPB formation but does not change the pathway. Indeed, no critical density of vesicles is observed and the curve shape is similar to those visualized for buffer **B4**. In fact, we have shown that the ionic strength is the key parameter for SPB formation via pathway 1 based on a critical density of adsorbed intact vesicles.

On the basis of these arguments, we arrive at an understanding of how different buffers influence the pathway of SPB formation as a function of buffer composition. The kinetics of the process can be tuned by the nature of the buffer (Tris·HCl or HEPES) and calcium ions while the pathway of bilayer formation is controlled by the sodium chloride. All these parameters are able to influence the vesicle−surface interactions and the vesicle−vesicle interactions responsible for the rupture of vesicles. Thus, intact vesicle can accumulate on the surface (low interactions and vesicle stabilization) or vesicles can break at low coverage (strong interactions and vesicle destabilization) as a function of the strength of the interactions involved.

These results on buffer composition were also analyzed using a ΔD versus Δf representation (Figure 1S, see Supporting Information).

1.2. Influence of pH on SPB Formation. This parameter controls the charge density of the SiO_2 surface and the nature (acidic or basic) of the lipids. From the pH studies, it turns out that SPBs are formed in any case except at high pH where the SiO_2 layer on the quartz is etched away (parts A and A* of Figure 2). It is known that SiO_2 dissolves at pH values above 10.[39] In a pH range of 3−10, the functional headgroups of the zwitterionic lipids are not sensitive to the pH. For pH values between 3 and 7, SPB formation was observed without passing through a critical density of vesicles on surface (Θ_c). This suggests strong vesicle−surface interactions destabilizing the vesicle structure and inducing their rupture. The supported bilayers seem to be formed via pathway 2. This behavior could be explained by the low degree of ionization of the SiO_2 surface in this range of pH values.[39] The experiments performed at pH values between 7.4 and 10 follow pathway 1. With increase of the pH value, the minimum in Δf decreases and the maximum in ΔD increases. These results suggest that more intact vesicles adsorb on the SiO_2 surface as a function of pH until Θ_c is reached. Thus, we can postulate that the SiO_2 surface seems to stabilize the vesicles as a function of its degree of ionization.

These results are in accordance with the data reported by Boxer et al. where they showed SPB formation with eggPC vesicles in the same range of pH using FRAP.[8] However, in the present work, we have shown for the first time that the pH controls the pathway and the kinetics of bilayer formation. While at acidic pH (below 7) vesicle rupture occurs at low vesicle coverage (pathway 2), at basic pH (above 7) vesicle rupture takes place at high vesicle coverage defined by Θ_c (pathway 1). As sodium chloride, pH is able to tune the vesicle−vesicle interactions and the vesicle−surface interactions controlling the SPB formation process.

1.3. Influence of Vesicle Concentration on SPB Formation. From parts B and B* of Figure 2, one can conclude that the kinetics of bilayer formation is concentration dependent. Indeed, the time needed to reach the critical density of the vesicle on the surface (Θ_c) and the time needed to form the SPB decrease with concentration. The kinetics of SPB formation can be illustrated by plotting either the frequency variation versus the exposure of the surface to vesicles (time $\times$ concentration) (Figure 4A) or ΔD versus Δf (Figure 4B).

Figure 4A shows that the entire SPB formation process does not scale exactly with the exposure of the surface to vesicles. The time needed to reach Θ_c (t_{Θ_c}) decreases with concentration increase. Above 0.1 mg/mL, the spreading process is fast, while for concentrations lower than 0.05 mg/mL it is very slow. Thus, these results suggest that the membrane formation is time dependent. However, the pathway of SPBs formation (pathway 1) remains independent of the vesicle concentration as depicted by the classical shape of the curves. The critical density of vesicles on SiO_2 (Θ_c), defined by the peak height in frequency, does not vary with the adsorption rate. This behavior, already described by Kasemo, indicates that the vesicle rupture begins at a critical density of vesicles on the surface.[40] However, the concentration dependence observed in the present work is distinctly different to experiments of Kasemo whose results were obtained with different lipids and with different experimental conditions. They showed that the entire adsorption process is essentially time independent and that the vesicle fusion and rupture are driven by the adsorption of vesicles from solution. This behavior was illustrated by the superposition of all the

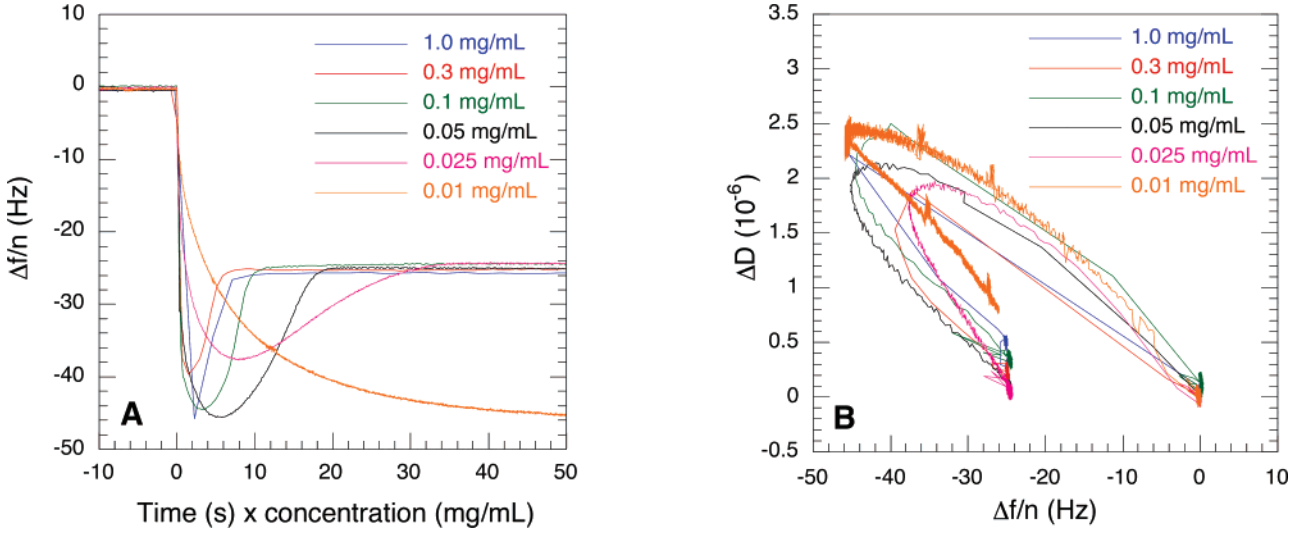


Figure 4. (A) SPB formation illustrated by the frequency shift versus exposure of surface (time × concentration) to different vesicle concentrations (DMPC, pH = 7.4, 33 °C): 0.01, 0.025, 0.05, 0.1, 0.3, and 1 mg/mL. By increase of the concentration, the minimum in $f(\Theta_c)$ is reached faster. For concentrations lower than 0.05 mg/mL, the process is very slow. (B) Plots of D versus f for the QCM-D data of parts B and B* of Figure 2 are represented. QCM-D data show the same behavior for all concentrations indicating SPB formation. Starting and ending values for f and D are similar in each case.

concentration curves. This suggests that in the case discussed here vesicles in solution play a significant role in the SPB formation pathway. That means that the pathway kinetics is controlled by the number of vesicles in solution which is dependent on the vesicle concentration. This difference in concentration dependence could also be interpreted by the presence of calcium in our study. The binding efficiency of calcium ions, known to accelerate the kinetics of bilayer formation by increasing the vesicle−surface interactions and the vesicle−vesicle interactions, increases with vesicle concentration.

$\Delta D/\Delta f$ plots (Figure 4B) allow the elimination of time effects and permit the elucidation of mechanistic details directly as a function of vesicle concentration. The superposition of the curves shows that the path traced out through the $f-D$ plane during the SPB formation process is independent of concentration of vesicles in solution and independent of how fast the progression of low to high coverage occurs. Data shown here, in good agreement with the experiments by Kasemo, suggest that the system passes always through the same set of states even for changing the kinetics by 2 orders of magnitude.

1.4. Influence of Temperature on SPB Formation. Here we investigate whether the temperature with respect to T_m has an influence on the fusion pathway. To answer this question, a temperature study around the T_m of a lipid mixture was performed and compared with a single lipid, DMPC. Recently, Kasemo et al. have shown that the transformation from vesicles to SPB is activated by temperature in the fluid state.[9] By decrease of the temperature, the time needed to reach the surface critical coverage (Θ_c) increases. The effect of vesicle rigidity on rupture process driven by electrostatic interactions has been shown by Janshoff et al.[12] They studied the adsorption of negatively charged vesicles (POPG/POPC and DPPG/DPPC) on a positively charged surface. They demonstrated using thickness shear mode (TSM) resonators that a higher percentage of PG is needed in DPPG/DPPC vesicles (more than 70%) than in POPG/POPC vesicles (30−50%) to induce the rupture of the vesicles. The results presented here demonstrate that the rigidity of vesicle membrane (⇒ mobility of individual lipids) controls the SPB formation. This rigidity can be changed by the lipid composition of the membrane. We started our study with pure DMPC (T_m value of 24 °C) that enables us to vary easily the temperature range from 15 to 33 °C. To slightly increase the T_m value and the rigidity of vesicles, different mixtures of DMPC/DPPC were prepared. DPPC possesses a T_m of 41 °C that is 4 °C higher than the temperature used for the experiments described here. As such, the DPPC lipids were mostly in the gel state. However, micro-DSC measurements have shown a perfect miscibility with DMPC in any mixture and a decrease of the T_m with increasing percentage of DMPC, as already reported in the literature.[30] As a consequence, all mixtures studied here (DMPC/DPPC 70/30 and 50/50) were in the fluid state at 37 °C (T_m of 29 and 33 °C, respectively).

At 37 °C, above the T_m, the transformation from vesicles to SPB via pathway 1 is observed in all cases. At 24 °C, close to the T_m value of the studied mixtures, vesicles started to rigidify and few of them stayed intact on the surface. By increasing the proportion of DPPC present in the mixture, the percentage of intact vesicles on SiO_2 increases (Figure 3). The preparation of vesicles composed of a lipid mixture leads to the formation of heterogeneous vesicles in lipid composition: vesicles contain a different percentage of DPPC. This has a direct influence on the rigidity and on the phase transition temperature (spreading of the micro-DSC peak compare to pure DMPC) of the individual vesicle. As a consequence, the rupture potential of these vesicle populations is different. This argument can explain the higher frequency values at Θ_c with higher proportion of DPPC and the shift of the minimum in frequency to higher time value.

At 15 °C, below the T_m, the rigidity of vesicles increases and the experimental curves suggest a low percentage of SPB regions on surface surrounded by a majority of intact vesicles. This rigidity induces low contact area between surface and vesicles inhibiting the rupture process. Our findings support a proposition by Kasemo, namely, that a stabilization of the intact vesicles on the surface by the surrounding SPBs occurs. This interpretation explains the variation of the expected frequency shift for a bilayer upon temperature of the experiment decreases.

ΔD versus Δf representation was also used to analyze these results on temperature (Figure 2S, see Supporting Information).

Plotting independently the frequency shift after stabilization (Δf_{stab}) versus temperature and the time needed to reach Θ_c (t_{Θ_c})

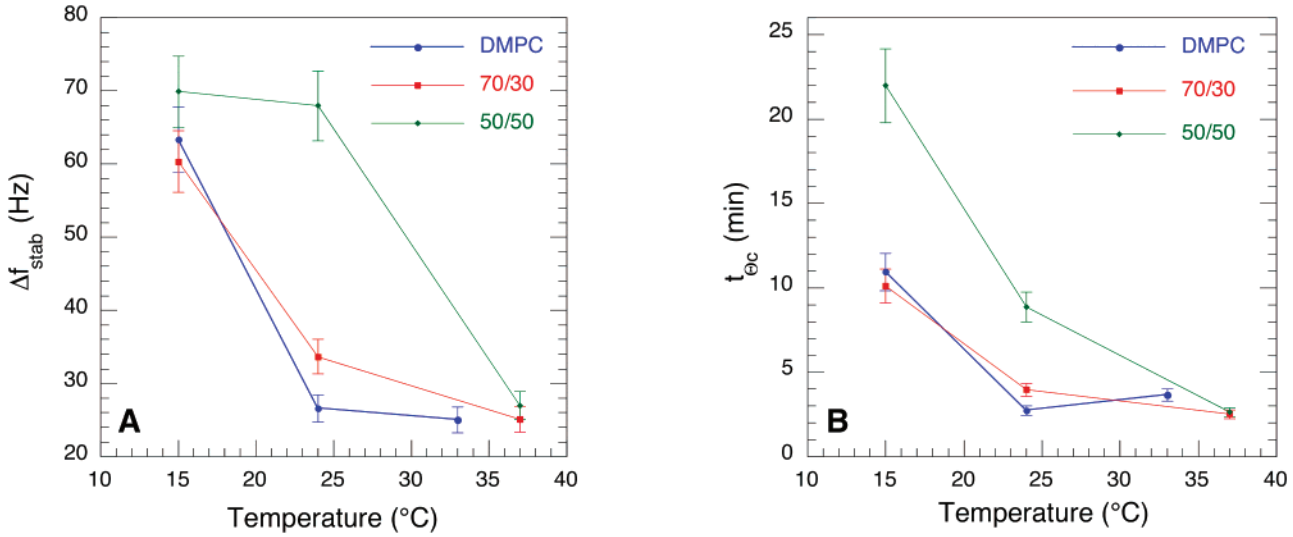


Figure 5. Plots of Δf_{stab} (A) and $t_{\Theta c}$ (B) as a function of temperature obtained from Figure 3 (DMPC, 0.05 mg/mL, pH = 7.4, 33 °C). These results show that distinction between SPB formation and adsorption of intact vesicles can be observed as a function of the temperature.

versus temperature illustrates the formation of SPB and/or the presence of intact vesicles as a function of the temperature (Figure 5).

In the Figure 5B, similar t_{Θ_c} values (around 3−4 min) are observed in all cases above the T_m. At 24 and 15 °C, the mixture 70/30 behaves like pure DMPC while the 50/50 mixture shows a different behavior. In fact, the latter at 24 °C has a comparable t_{Θ_c} as for DMPC at 15 °C suggesting a similar rigidity. Qualitative evaluation of these results suggests an Arrhenius-like behavior.[41,46]

Figure 5A shows clearly the influence of temperature on SPB formation depending on the lipid composition and the T_m. Above the T_m, typical values for supported membranes (around 26 Hz) are obtained. At 24 °C, only DMPC keeps the same behavior while the 70/30 mixture deviates slightly and the 50/50 mixture shifts to high-frequency values (around 70 Hz). At 15 °C, DMPC and 70/30 mixture behave like the 50/50 indicating a majority of intact vesicle on surface. Using these curves, we are able to visualize the T_m value of the lipid mixture and when the behavior is changing. In addition, information about the lipid mixture state (fluid or rigid) is given. The results shown here demonstrate that the phase transition temperature of a lipid mixture has a critical effect on SPB formation. Complete bilayer formation on SiO_2 occurs only for temperature above the T_m of the lipid mixture studied.

Conclusions

Our studies have led to new qualitative and quantitative information to understand the SPB formation process for lipid mixtures having a high T_m. Two mechanistic pathways could be distinguished as a function of the experimental parameters.

First, we find that buffer composition can influence the SPB formation on SiO_2. The presence of salt such as sodium chloride and calcium chloride is required to induce and accelerate the SPB phenomenon via pathway 1. Thus, a critical density of intact vesicles on the surface (Θ_c) is observed before the transformation from vesicles to a supported lipid bilayer occurs. We have also shown that the buffer pH has an effect on the pathway of SPB formation in the case of zwitterionic lipids. Below a pH of 7.4 pathway 2 is observed while between a pH of 7.4 and 10 pathway 1 is dominant.

All the previous observations on buffer composition and pH are of course the consequence of a subtle balance among the interactions between the bilayer and the support (van der Waals, electrostatic, hydration, and steric forces) that control the mechanistic pathway.

Further, we have demonstrated that the vesicle concentration has an effect on the kinetics of SPB formation without modifying the mechanistic pathway. Θ_c is reached faster with increasing concentration. No trapped vesicles into formed SPB could be observed even at high concentration. This study has shown that the kinetics of SPB formation are dependent on the concentration for the case of DMPC/DPPC mixtures in contrast to lipids having a low T_m.

Afterward, we have shown that the temperature difference between T_m and the experimental temperature is crucial for SPB formation. Above and around T_m, transformation of vesicles to SPB via pathway 1 is observed, while below, a majority of intact vesicles surrounded by some SPBs is obtained. In this situation, temperature controls the rigidity of the vesicles influencing both the energetic barrier and the contact area to overcome the formation of SPB. Temperature decreases this barrier and activates SPB formation process. On the basis of these results, the first steps in understanding the behavior of lipid mixtures under T_m on SiO_2 surface were undertaken. It seems that ideal supported membranes have to be prepared at least 10 °C above the melting temperature of the corresponding lipid mixture. One can thus conclude that mobile lipids in the vesicle membrane favor dynamic membrane processes including rupture, fusion, etc. while the immobile lipids render this processes more difficult. The QCM-D technique allows us to determine in situ changes in the pathway as a function of the experimental temperature.

From a practical point of view, we have identified the best experimental conditions (buffer, concentration, and temperature) that are ideally suitable for investigating DMPC/DPPC mixtures, possessing a high phase transition temperature, to form SPB.

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Supporting Information Available: Additional information on buffer and temperature studies given as ΔD versus Δf plots.

This material is available free of charge via the Internet at http://pubs.acs.org.

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