Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1617 (2003) 39–51
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Preferential orientation of an immunoglobulin in a glycolipid monolayer controlled by the disintegration kinetics of proteo-lipidic vesicles spread at an air–buffer interface

Agnès P. Girard-Egrot[a,*], Stéphanie Godoy[a], Jean-Paul Chauvet[b,1], Paul Boullanger[c], Pierre R. Coulet[a]

[a] *Laboratoire de Génie Enzymatique et Biomoléculaire, UMR 5013/EMB2-CNRS/UCBL, Université Claude Bernard Lyon 1, 43 Bvd du 11 novembre 1918, Villeurbanne cedex F-69622, France*
[b] *Ingénierie et Fonctionnalisation des Surfaces, UMR 5621-CNRS/ECL, Ecole Centrale de Lyon, 36 Avenue Guy de Collongue, Ecully cedex F-69134, France*
[c] *Laboratoire de Chimie Organique 2, UMR 5181-CNRS/UCBL, Université Claude Bernard Lyon 1, Ecole Supérieure de Chimie Physique Electronique de Lyon, 43 Bvd du 11 novembre 1918, Villeurbanne cedex F-69622, France*

Received 30 July 2002; received in revised form 28 July 2003; accepted 11 September 2003

This paper is dedicated to the memory of Pr Jean-Paul Chauvet

Abstract

The insertion of immunoglobulin (IgG) in a glycolipid monolayer was achieved by using the ability of new proteo-glycolipid vesicles to disintegrate into a mixed IgG–glycolipid interfacial film after spreading at an air–buffer interface. The interfacial disintegration kinetics was shown to be directly dependent on the initial vesicle surface density and on the buffer ionic strength. The presence of the immunoglobulin in the glycolipid film was displayed by an increase of the lateral compressibility (Cs) during monolayer compression. Cs magnitude modifications, due to the antibody effect on the monolayer packing, decreases as the spread vesicle density increases. At interfacial saturation, the lateral compressibility profile becomes similar to that of a control monolayer without antibody. However, the careful analysis of the mixed monolayer after transfer by Langmuir–Blodgett technique (ATR-FTIR characterisation, enzyme immunoassociation) clearly demonstrated that the antibody was still present in such conditions and was not completely squeezed out from the interface as compressibility changes could have meant. At nonsaturating vesicle surface density, IgG molecules initially lying in the lipid matrix with the Y-shape plane parallel to the interface move to a standing-up position during the compression, leading to lateral compressibility modifications. For a saturating vesicle surface density, the glycolipid molecules force the IgG molecules to directly adopt a more vertical position in the interfacial film and, consequently, no lateral compressibility modification was recorded during the compression.

Keywords: Vesicle spreading; Interfacial monolayer; Acetylcholinesterase; Glycolipid; Monoclonal antibody; Protein orientation

1. Introduction

Among the numerous functions mediated by the biological membranes, processes such as recognition, sensing and information exchange could be involved in practical applications. In this context, biosensors are operational tools that mimic some functions of the cell membrane such as recognition or transduction of biological signals. However, the performance of biosensors and other bioelectronic devices mainly depend on the properties of the bioactive layer associated with the transducer. The integration of sensitive layers organised at the nanoscale scale is promising for

Abbreviations: A, area; AChE, acetylcholinesterase; ATR-FTIR, attenuated total reflection–Fourier-transform infrared; au, arbitrary unit; BSA, bovine serum albumin; DTNB, (5,5′ -dithio-bis(2-nitrobenzoic acid); EU, Ellman's unit; I, ionic strength; IgG, immunoglobulin G; LB, Langmuir–Blodgett; LC, liquid condensed; LE, liquid expansed; MLV, multilamellar vesicle; *t*, time; VSD_{init}, initial vesicle surface density; π, surface pressure; π_T, transfer surface pressure

* Corresponding author. Tel.: +33-47-243-8532; fax: +33-47-244-7970.
E-mail address: agnes.egrot@univ-lyon1.fr (A.P. Girard-Egrot).
[1] Deceased (January 12, 2003).

0005-2736/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2003.09.001

further developments of nanobiosensors with signal detection at the molecular level.

Langmuir–Blodgett (LB) technology offers an efficient opportunity to develop well-organised lipidic nanostructures as biomimetic membranes, and many works have proposed the association of biocompounds (generally enzymes) to LB films for new biosensor developments [1–17]. However, in these approaches, enzyme molecules were more often adsorbed on preformed lipidic LB films, leading to a random immobilisation and to a weak association. Until now, the main problem to be solved is the control of the enzyme retention in a defined orientation, like in biological membranes where the protein association in/on the lipidic leaflets determines their own orientation for an optimal functionality.

In order to develop functionalized biomimetic nanostructures, we propose to insert a monoclonal immunoglobulin (IgG) in LB films, to play the role of an anchor able to ensure an orientated positioning of the enzyme at the surface of the lipidic matrix after specific recognition.

Interfacial disruption of vesicles injected in the subphase [18–20] or directly spread at an air–buffer interface allows preparing an interfacial lipid film [21–25]. In our group, we proposed earlier to use the fusion of proteo-vesicles spread at an air–buffer interface to prepare proteo-lipidic sensitive layers [26,27]. Recently, this approach appeared very efficient to bring and to retain immunological proteins coming from an ascitic fluid at the air–buffer interface [28]. This methodology is an alternative way as the one recently proposed to form a stable enzyme Langmuir film under subphase conditions improving the interfacial protein stability [29,30].

This paper focuses on the insertion of a noninhibitor monoclonal immunoglobulin in a neutral synthetic glycolipid matrix by spreading proteo-glycolipid vesicles at an air–buffer interface. This antibody is directed against the acetylcholinesterase (AChE, EC 3.1.1.7) monomer of the *Bungarus fasciatus* venom. In a previous study, we reported that these proteo-glycolipid vesicles were able to rapidly disintegrate at an air–buffer interface to form a true mixed IgG-glycolipid interfacial film [31]. The interactions occurring between the glycolipid and the glycoprotein (IgG) in the vesicle membranes prior to the interfacial film formation, appeared strong enough to keep IgG inserted in the glycolipid film during the vesicle disintegration and to promote its transfer together with the lipidic film by vertical deposition [32]. The ability to retain active acetylcholinesterase at the surface of the IgG-glycolipid LB film after immunoassociation has been recently demonstrated [33].

In the present work, we have investigated the initial orientation of IgG molecular plane in the glycolipid matrix through a detailed analysis of the lateral compressibility modifications versus the initial vesicle surface density (VSD_{init}) spread on a phosphate buffered subphase at different ionic strengths. VSD_{init} is defined as the ratio of the vesicle spread amount on the initial available trough area. This parameter, which directly controls the kinetics of the surface film formation, appears to play a crucial role in the original positioning of IgG molecular plane in the lipidic matrix before the monolayer compression. By a minute control of the VSD_{init} parameter, the initial orientation of the Y-shaped immunoglobulin molecule can be directly controlled through the disintegration kinetics of the proteo-glycolipid vesicles spread at the air–buffer interface.

2. Materials and methods

2.1. Materials

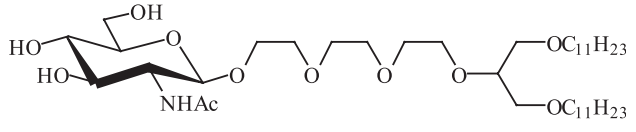
Solvents and all chemical reagents were of analytical grade and used without further purification. The glycolipid, 10-undecyloxymethyl-3,6,9,12-tetraoxa-tricosyl 2-acetamido-2-deoxy-β-D-glucopyranoside[2] was synthesised as previously reported [34]. The monoclonal immunoglobulin directed against acetylcholinesterase (AChE) of *B. fasciatus* venom, extracted from a mouse ascitic fluid, was generously supplied by Dr. Grassi (SPI, CEA Saclay, Gif sur Yvette, France). It was purified by protein A chromatography onto Protein A HyperD F (BioSepra, France) as previously described [31]. The *B. fasciatus* AChE monomer was a generous gift of Dr. Bon (Institut Pasteur de Paris, Unité des venins, France). *S*-acetylthiocholine iodide, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) and IgG free-bovine serum albumin (BSA) were purchased from Sigma-Aldrich Chimie (St-Quentin Fallavier, France). Ultrapure water (18.2 MΩ cm) from a milli-Q four-cartridge purification system (Millipore, France) was used to prepare phosphate buffered subphases and other buffer solutions.

2.2. Methods

2.2.1. Glycolipid and immunoglobulin–glycolipid vesicle formation

The glycolipid and proteo-glycolipid vesicles were formed as previously described [31]. Briefly, a dried glycolipid film was mechanically dispersed above the main transition temperature of the synthetic glycolipid, either in 1 ml of 10 mM phosphate buffer pH 7.4 to form glycolipid vesicles or in 1 ml of the purified antibody solution to form IgG–glycolipid vesicles. The final glycolipid concentration was 10 mg/ml and for the proteo-glycolipid vesicles, the protein–lipid molar ratio was 1:450. The multilamellar structure (MLV) and the mean diameter of the vesicles

2



appeared very similar to those previously described [31]. The vesicle concentrations were estimated at 9.8×10^{12} and 2.6×10^{13} vesicles/ml for glycolipid and IgG-glycolipid vesicles, respectively, assuming a mean diameter of 150 and 100 nm, respectively, and a five bilayer structure [31]. The vesicle suspensions were directly used for the spreading procedure and could be stored at 4 °C for 1 week without apparent modification of their spreading kinetics.

2.2.2. Interfacial film formation

The interfacial film was formed in a computerised KSV 3000 Langmuir–Blodgett trough (KSV, Finland) enclosed in a filtered air dry flow cabinet to avoid dust deposition. Phosphate buffer (pH 7.4) thermostated at 20 ± 0.5 °C was used as subphase. Two dimensions of troughs were used for the spreading procedures (772.5 and 382.5 cm^2). In order to vary VSD_{init}, 2, 5 or 10 μl of vesicle suspension were carefully deposited at the interface using a 50 μl SGE microsyringe. VSD_{init} was defined as the ratio of the amount of vesicle spread on the initial trough area. The subphase ionic strength (I) was modified by changing the phosphate concentration from 0.01 to 1.5 M and was calculated according to the following equation:

$$I = 1/2\Sigma[C_i z_i^2] \quad (1)$$

in which C_i and z_i are the molar concentration and the charge of each ionic species present in the medium, respectively. The kinetics of the surface film formation corresponding to the interfacial vesicle disintegration was followed by recording the surface pressure variations during a period of 35 min. The surface pressure (π) was measured using a platinum Wilhelmy plate attached to a sensitive balance with an accuracy of ± 0.3 mN/m. The zero of the time scale was ascribed to the beginning of the spreading procedure; 35 min after spreading, the interfacial films were compressed symmetrically at a rate of 15 cm^2/min. Due to the irreversible vesicle diffusion from the interface to the bulk phase during the spreading procedure, the glycolipid surface concentration (Γ) could not be exactly determined. Therefore, the surface pressure–area ($\pi - A$) diagram was expressed versus the total surface film area (A, in cm^2). The two-dimensional lateral compressibility of the monolayer (Cs) was directly calculated from the slope of π–A isotherm diagram by the KSV's software according to the following equation:

$$Cs = -1/A(\partial A/\partial \pi)_T \quad (2)$$

where A is the surface film area at the indicated surface pressure and π the corresponding surface pressure [35]. This interfacial parameter is expressed in m/mN and is often interpreted as its reciprocal form (Cs^{-1}), originally defined by Davies and Rideal [36] as the surface compressional modulus, which is a measurement of the compressional elasticity of the film.

2.2.3. Langmuir–Blodgett film deposition

The interfacial film was transferred by vertical Langmuir–Blodgett technique onto different solid supports. Germanium internal reflection parallelogram plates ($25 \times 10 \times 3$ mm, Spectra-Tech Inc., France), crystals for ATR-FTIR spectroscopy measurements, and calcium fluoride rectangular plates ($35 \times 9.5 \times 2$ mm, Sorem, France), support for Nomarski microscopic observations were used as substrates. They were thoroughly cleaned just prior use with an ionic detergent (Hellmanex II, Eurolabo, France), as described elsewhere [28]. For the transfer procedure, the germanium crystal was clamped parallel to the barriers and rapidly immersed into the aqueous subphase just before the compression in order to avoid any adsorption during the time elapsed for the surface film formation [37]. The interfacial film was then compressed up to the transfer surface pressure at a rate of 15 cm^2/min and one layer was deposited onto the germanium at the upstroke with a dipping rate of 5 mm/min. For the detection of the immunoaffinity of the antibody inserted in the transferred monolayer, a calcium fluoride plate was precoated with four layers of behenic acid spread onto a 10^{-2} M NaCl, 10^{-4} M $MnCl_2$ subphase [38], prior to the transfer of the mixed antibody–glycolipid monolayer as recommended elsewhere [39]. After transfer, the stability of the interfacial film was systematically checked during 15 min at the transfer surface pressure by relaxation experiment. The transfer ratio was calculated for each deposition from the surface film removed.

2.2.4. Infrared spectroscopy

Infrared spectra were recorded with a Fourier-transform infrared (FTIR) spectrometer (Model 510 M, Nicolet instruments, France) equipped with a DTGS room temperature detector. The instrument was continuously purged with dry air delivered by a Balston air purifier. Germanium ATR with a 45°-face angle was used as internal reflection element, yielding eight internal reflections. The ATR plates were considered exempt of contamination if the $\nu(CH_2)$ bands at ~ 2920 and ~ 2850 cm^{-1} completely disappeared in the ATR-FTIR spectrum (single beam mode). After monolayer deposition, the crystal was meticulously dried under a filtered dry air flow and put in a variable angle vertical ATR accessory (Model 300, Spectra-Tech Inc., France). Several spectra of 150 scans were collected in a single beam mode with a resolution of 4 cm^{-1}. After cleaning, backgrounds were recorded in the same way. All spectra reported here result from the subtraction of the background data measured with the clean germanium plate from those measured on the same germanium plate covered by the transferred film. The integration of peak areas proposed by the Nicolet's software allowed a quantitative comparison between the spectra. This integration value is given in arbitrary unit (au).

2.2.5. Detection of the immunoaffinity of the immunoglobulin in the transferred monolayer

The immunoaffinity of the antibody inserted in the mixed monolayer was detected after the transfer of two IgG-glyco-

lipid monolayers onto behenic acid precoated-CaF_2 plates. The enzyme immunoassociation was performed by immersion of the coated support in an AChE solution (30.36 EU/ml) in 0.1 M phosphate buffer at pH 7.4 containing 0.15 M NaCl and 1 mg/ml BSA, under gentle magnetic stirring. After an immunoreaction of 18 h at 4 °C, the plates were washed twice during 30 min in the same buffer. The activity of AChE bound on the solid plate was measured by means of the colorimetric Ellman's method [40] using 0.75 mM acetylthiocholine iodide as enzyme substrate, in the presence of 0.25 mM DTNB in 0.01 M phosphate buffer, pH 7.4. The hydrolysis of the enzyme substrate was monitored at 412 nm for 2 min in a Kontron-Uvikon 942 spectrophotometer with a cell thermostated at 25 °C. The spontaneous hydrolysis of the substrate was determined in the absence of the bound-enzyme support and was routinely subtracted. The AChE activity retained on the solid plate was expressed in Ellman's units/cm^2. One Ellman's unit (EU) was defined as the enzyme activity producing an absorbance increase of 1 unit (412 nm) at 25 °C, in 1 min, with 1 ml of medium and for an optical path length of 1 cm. The nonspecific adsorption of AChE was checked using a IgG-free glycolipid interfacial film formed by vesicle spreading and transferred under the same experimental conditions on a behenic acid-coated CaF_2 plate.

3. Results and discussion

3.1. IgG–glycolipid monolayer formation

Immunoglobulin–glycolipid interfacial film was formed by careful deposition of the vesicle suspension at the air–liquid interface (referred as vesicle spreading procedure). Fig. 1A exhibits the typical time-dependent variation of the surface pressure recorded after spreading of glycolipid (a) or IgG–glycolipid vesicles (b) onto a phosphate buffered subphase with a high ionic strength (I = 1.17). The increase of the surface pressure observed after vesicle spreading has been firstly reported by Verger and Pattus [21]. Now, such behaviour, corresponding to the kinetics of the surface film formation, has been intensively studied both experimentally and theoretically, for different sizes of phospholipidic vesicles [22,24,41–44]. For both types of glycolipid vesicles studied here, with or without antibody, the increase in the surface pressure has been attributed to the formation of an interfacial film consecutive to the disintegration of the vesicle membranes at the interface [26,32], and by applying theoretical analysis proposed by Ivanova, Panaiotov and coworkers [42,43,45], we have previously demonstrated that these glycolipid vesicles disintegrate into a true monomolecular film, even in the presence of the anti-AChE immunoglobulin [31]. For the proteo-glycolipid vesicles, the surface pressure increase is higher than that observed for glycolipid vesicles, suggesting that more material is maintained at the interface during the opening process. This behaviour has been attributed to the modification of the membranous system, due to the insertion of the protein molecules into the vesicle membranes [31]. Such an insertion weakens the structural organisation of the glycolipid bilayers and introduces a greater interfacial instability.

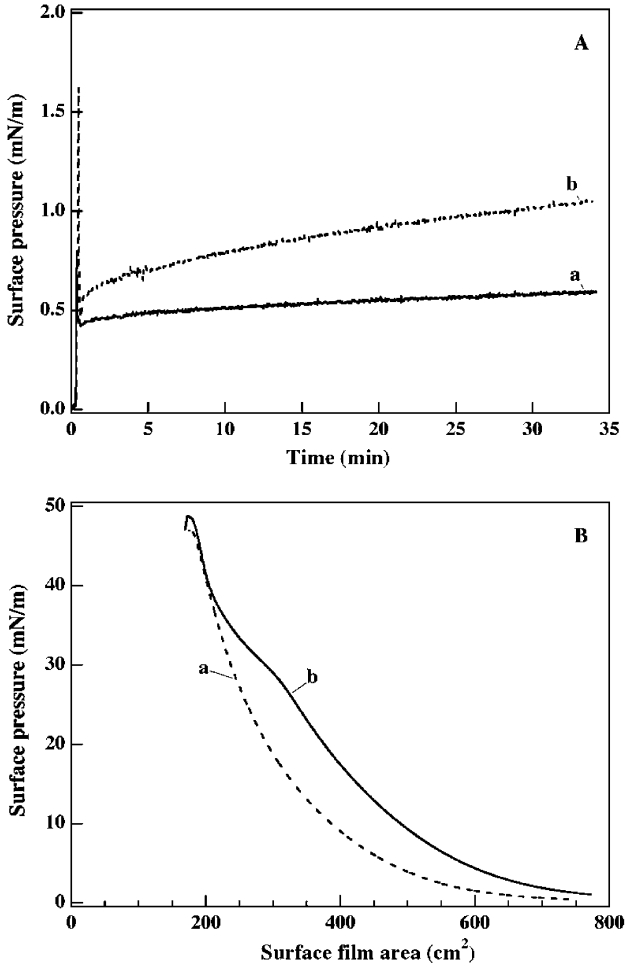


Fig. 1. Formation of the interfacial film by liposome spreading. (A) Kinetics of the surface film formation recorded for 35 min after spreading of glycolipid (a) or IgG–glycolipid (b) vesicles on a phosphate buffered subphase (I = 1.17). $VSD_{init} = 6.34 \times 10^7$ vesicles/cm^2 for glycolipid (a) and 6.73×10^7 vesicles/cm^2 for IgG–glycolipid (b). Zero on the time scale corresponds to the beginning of the spreading. Curve b reaches a constant surface pressure after a delay period just higher than 35 min. (B) Corresponding π–A Isotherms of the pure (a) and mixed (b) interfacial film. Monolayer compression was performed 35 min after spreading.

The π–A isotherms recorded 35 min after spreading, when the surface pressure has reached a quasi-equilibrium value, are presented in Fig. 1B. The presence of the immunoglobulin in the mixed monolayer was evidenced by the increase in the surface film area for a given surface pressure up to relatively high π values (~ 41 mN/m).

3.2. Influence of VSD_{init} on the in-plane elasticity of the mixed monolayer

The lateral compressibility of monolayers (Cs), and more directly its reciprocal form (Cs^{-1}), i.e. elastic moduli of area

compressibility [46], is related to the in-plane elasticity of the monolayer. Compared with conventional surface pressure isotherms, the surface compressibility can be used to characterise more carefully the lipid phase transition during the monolayer compression [47]. According to Ihalainen and Peltonen [48], the π–Cs curve is more sensitive to intermolecular interactions and especially to their changes. In our case, the expanding effect of the immunoglobulin upon the glycolipid monolayer has been analysed by the variations of the two-dimensional monolayer compressibility (Cs) as a function of lateral packing pressure (Fig. 2). As expected for lipidic monolayers displaying a liquid expanded π–A isotherm (as is the case for the glycolipid monolayer [28]), the lateral compressibility of the pure glycolipid monolayer (reference curve *f*) decreases with increasing the surface pressure until the collapse is approached [46,49]. The maximal compressibility observed at 32 mN/m for the mixed interfacial film is characteristic of the fluidising effect of the protein to interfacial properties of the glycolipid monolayer; it expresses a decrease of the lateral packing density. The higher the magnitude in Cs variation occurred, the greater the effect of the protein was. The effect of VSD_{init} on the in-plane elasticity of the mixed proteo-glycolipid monolayer is displayed in Fig. 2 (curves a–e). The magnitude of Cs increase (around 30 mN/m) lessens with the increase of the spread amount of proteo-glycolipid vesicles. For the highest VSD_{init} (68.0×10^7 vesicles/cm^2, curve e), the Cs profile became similar to that obtained for a pure glycolipid monolayer. This VSD_{init} corresponds to the interfacial saturating density since a higher VSD_{init} gave practically the same equilibrium surface pressure value after disintegration (data not shown). A larger VSD_{init} induces a larger saturation of the interface

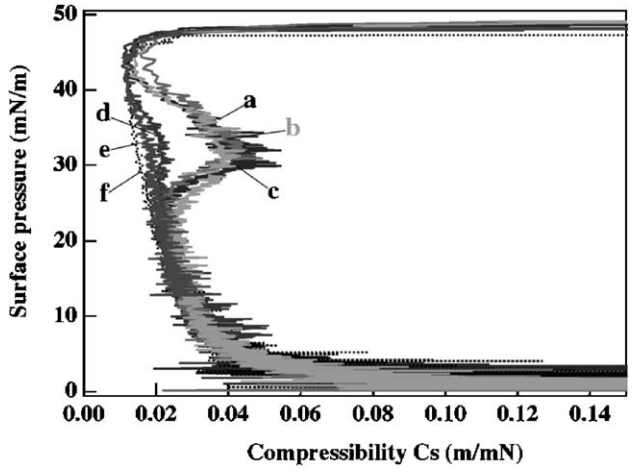


Fig. 2. Surface pressure (π) vs. two-dimensional compressibility (Cs) curves of mixed monolayers formed by spreading of IgG–glycolipid vesicles onto a phosphate-buffered subphase (I = 1.17) for different VSD_{init}. (a) 6.73×10^7 vesicles/cm^2; (b) 13.6×10^7 vesicles/cm^2; (c) 16.8×10^7 vesicles/cm^2; (d) 33.8×10^7 vesicles/cm^2; (e) 68.0×10^7 vesicles/cm^2. (f) corresponds to the π–Cs curve of pure glycolipid monolayer formed by spreading glycolipid vesicle suspension ($VSD_{init} = 6.34 \times 10^7$ vesicles/cm^2). (The same curve was obtained for other VSDs).

and a less pronounced effect of the protein. For a proteolipidic monolayer, the lateral compressibility variation occurring during the compression can be ascribed either to an expulsion or to a molecular reorientation of the protein in the lipidic matrix. Recently, by analysing mixed cholesterol–phospholipid monolayers, Keller [50] has reported that the compressibility was sensitive to the orientation of molecules at the interface, and that monolayer compressibility could be used as a sensitive tool to investigate interactions between cholesterol and phospholipids. In order to understand why the magnitude of the compressibility variations was inversely related to VSD_{init}, we have systematically investigated the lateral compressibility of the mixed monolayer in relation with the interfacial disintegration kinetics of the IgG-glycolipid vesicles, on various phosphate-buffered subphases at different ionic strengths. Thus, varying the subphase ionic strength could modulate the interfacial protein expulsion that could occur during the compression.

3.3. Kinetics of interfacial disintegration of IgG–glycolipid vesicles, lateral compressibility of the mixed monolayer under subphases of various ionic strengths and for different VSD_{init}

IgG–glycolipid vesicles have been spread onto phosphate buffered subphases of ionic strengths ranging from 3.5 to 0.023. Fig. 3 shows the interfacial disintegration kinetics of the IgG–glycolipid vesicles and the corresponding compressibility of mixed monolayers for different VSD_{init}. As expected, decreasing the subphase ionic strength resulted in a decrease of the magnitude of both phenomena, i.e. kinetics of the surface film formation and Cs variations. A decrease in the ionic strength could not prevent the irreversible diffusion of vesicles in the subphase and, consequently, the actual number of vesicles retained at the interface and able to disintegrate was lowered. Actually, the detailed analysis of the π–Cs curves revealed that the two-dimensional compressibility Cs evolved inversely with VSD_{init}, even at low ionic strengths.

A phenomenological correlation between the Cs profile and the pattern of the kinetics of the surface film formation can be outlined. When the proteo-glycolipid vesicles disintegration process presented an obvious kinetic pattern, Cs variations were small for the highest subphase ionic strengths or completely disappeared for the smallest ones (see curves d, e, Fig. 3). When the disintegration process presented low kinetic effects (high ionic strengths) or when surface pressure reached quasi-instantaneously a constant value after the initial peak (small ionic strengths), the largest Cs variations were recorded during the compression (see curves a–c, Fig. 3).

The kinetics of the surface film formation of the glycolipid monolayer can be interpreted on the basis of previous studies, which have theoretically and experimentally analysed the monolayer formation from spreading of phospholipid liposomes [23,24,42,43]. The formation of an

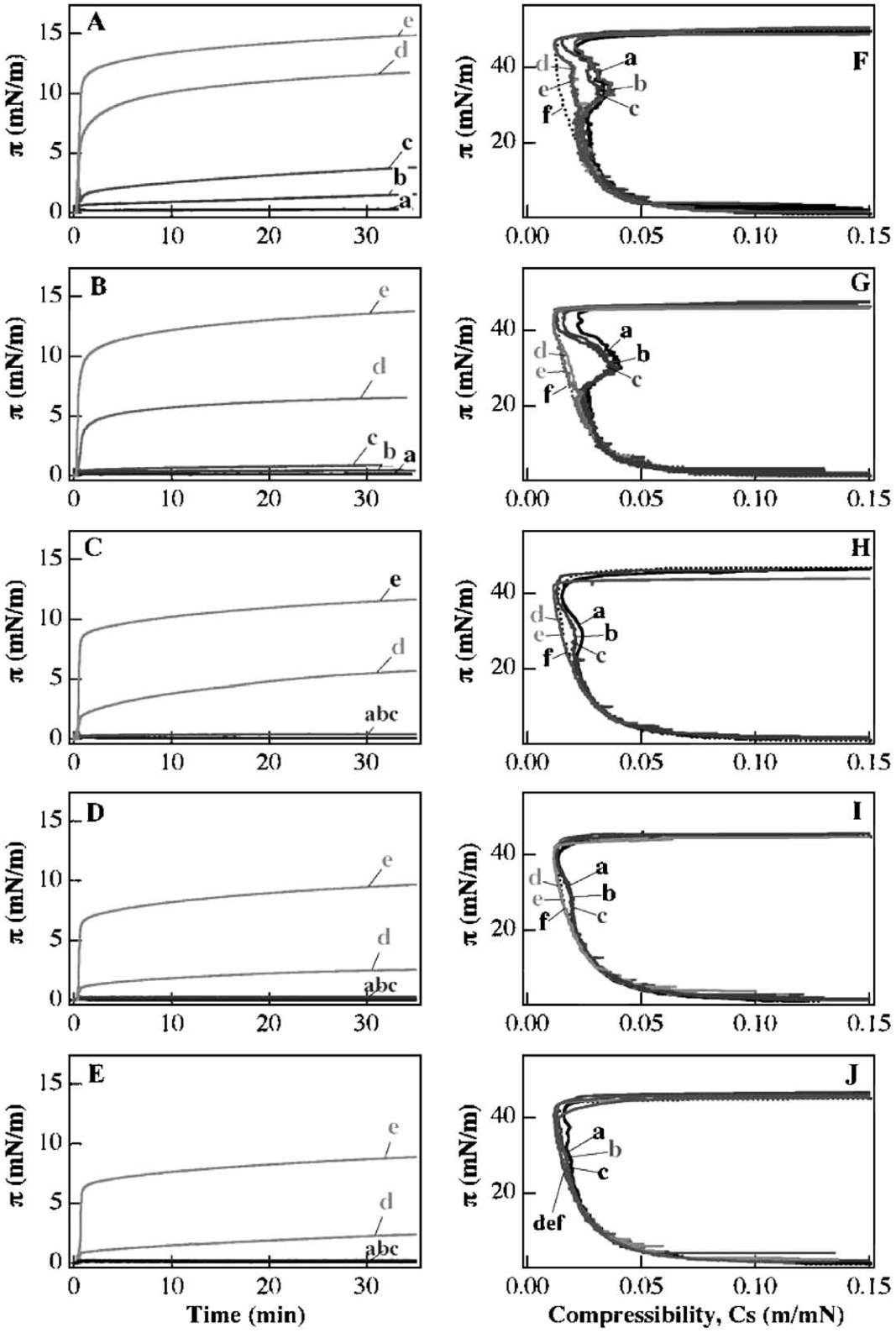


Fig. 3. Interfacial disintegration kinetics of IgG–glycolipid liposomes (A–E) and two-dimensional compressibility (Cs) of the resulting mixed monolayer (F–J) formed under various subphase ionic strengths for different VSD_{init}. Subphase ionic strength (I) was modified by changing the phosphate concentration. (A, F) $I=3.5$; (B, G) $I=1.17$; (C, H) $I=0.23$; (D, I) $I=0.12$; (E, J) $I=0.023$). VSD_{init}: (a) 6.73×10^7 vesicles/cm^2; (b) 13.6×10^7 vesicles/cm^2; (c) 16.8×10^7 vesicles/cm^2; (d) 33.8×10^7 vesicles/cm^2; (e) 68.0×10^7 vesicles/cm^2. (f) corresponds to the π–Cs curve of pure glycolipid monolayer formed by spreading glycolipid vesicle suspension. (The profile was identical whatever VSD_{init}).

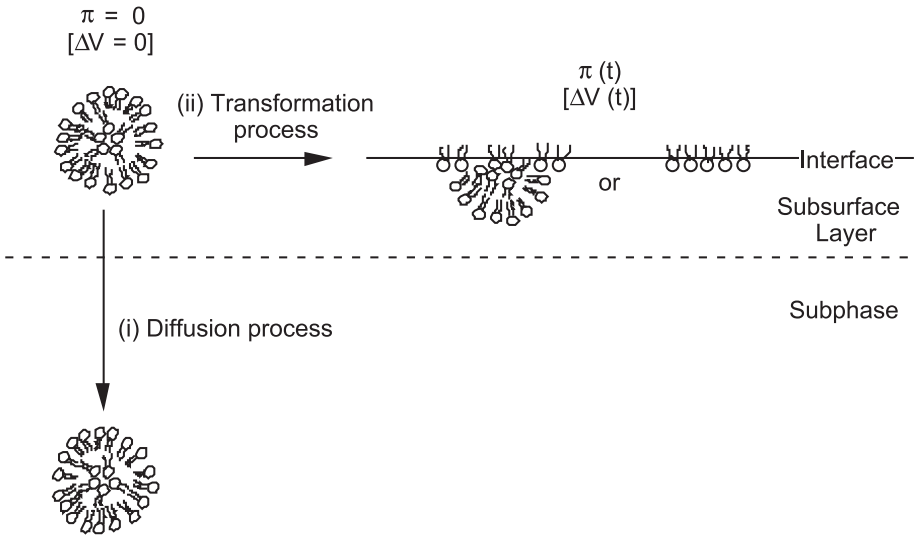


Fig. 4. Competitive processes involved in the interfacial film formation from liposome spreading. On this scheme, only the opened structures are assumed to be surface active [42,43].

interfacial film from vesicle spreading is a part of a process composed of two competitive steps occurring simultaneously (Fig. 4): (i) an irreversible diffusion of closed vesicles toward the bulk subphase and (ii) an irreversible transformation of closed vesicles into an interfacial film. The liposome suspension initially spread at the air–buffer interface is assumed to form a subsurface layer (solution zone underlying the interface) with a defined thickness (L) [23,24,42,43]. Depending on the thickness, the kinetics of one of both processes becomes dominant, and two borderline cases can be described (Fig. 5). (i) If large amounts of vesicles are spread (saturating conditions), the kinetics is controlled by the transformation process and the kinetic curve can be described by a Langmuir-like adsorption kinetic equation [43]. (ii) If small amounts of vesicles are spread (nonsaturating conditions), a diffusion-controlled kinetics can be described by solving the second Fick's equation at boundary conditions [42]. By applying the theoretical analysis developed by these authors, we have previously demonstrated that under saturating spreading conditions, the kinetics of surface film formation obtained by spreading glycolipid vesicles was fully controlled by a

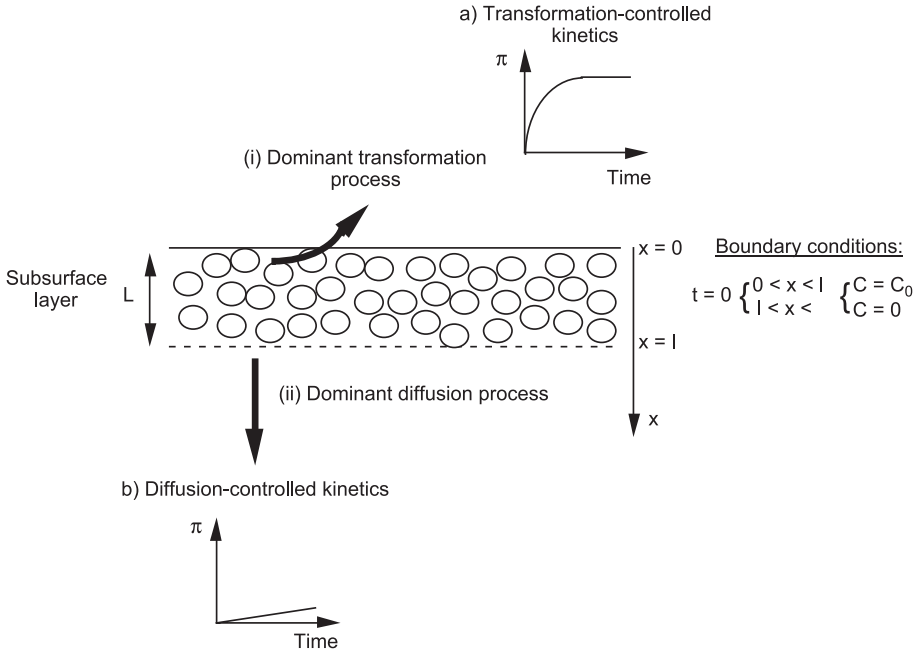


Fig. 5. Borderline cases of the kinetics involved in the liposome surface transformation. (a) Transformation-controlled kinetics; (b) diffusion-controlled kinetics [24,43].

transformation process, even in the presence of immunoglobulin [31]. In this study, we report on the role of the subphase ionic strength over the kinetics pattern. For high VSD_{init} (e.g. 33.8×10^7 vesicles/cm^2, curve d, Fig. 3), the kinetics initially controlled by transformation process at high ionic strength (Fig. 3A) became controlled by an intermediate process at low ionic strengths (Fig. 3D,E). In these latter cases, the vesicle diffusion started to influence the overall phenomenon. For low VSD_{init} (e.g. 6.73×10^7, 13.6×10^7 and 16.8×10^7 vesicles/cm^2, curves a, b and c, Fig. 3), the kinetics was entirely controlled by the diffusion process, mostly at low ionic strengths. Therefore, the relationship between the kinetics of vesicle disintegration and the variations of the monolayer compressibility could be interpreted by comparison of the curves reported in Fig. 6. These latter were registered at constant ionic strength ($I = 1.17$) and for two different VSD_{init} (16.8×10^7 vesicles/cm^2, curves a and 33.8×10^7 vesicles/cm^2, curves b). When the transformation process predominantly controls the kinetics of the surface film formation (curves b), the expanding effect of the immunoglobulin on the monolayer properties is less pronounced than that observed when the vesicle diffusion process predominantly controls the kinetics (curves a). The effect of the protein on the in-plane monolayer elasticity is then directly dependent on VSD_{init}.

3.4. Presence of IgG in the condensed film

The presence of the immunoglobulin in the condensed monolayer has been characterised by ATR-FTIR studies after transfer onto a solid germanium plate. In a first time, the monolayer was formed under nonsaturating conditions ($VSD_{init} = 16.8 \times 10^7$ vesicles/cm^2) for which spreading kinetics, mainly controlled by the diffusion step, ensured a large compressibility variation during the compression. The transfer of the monolayer was realised in the condensed state, far enough from the collapse surface pressure at the maximal compressibility. Depending on the subphase ionic strength, the transfer surface pressure was chosen in order to ensure the same packing to the transferred monolayers. Reference monolayers using pure glycolipid were transferred under the same conditions. With phosphate buffers of high ionic strengths, the high viscosity led to the subphase entrainment that could be observed, after transfer, by Nomarski microscopy (data not shown). Under this condition, strong phosphate buffer absorption bands masked the characteristic bands of glycolipid and protein molecules, thus hindering the fine analysis of ATR-FTIR spectra at high subphase ionic strengths. Fig. 7 shows the typical 1500–1750 cm^{-1} spectral region for the lowest ionic strength. This region corresponding to the Amide I (~ 1650 cm^{-1}) and Amide II (~ 1550 cm^{-1}) vibrations of the peptide bond enables to specifically characterise the presence of the protein. However, the glycolipid also displays the same two bands in this region (*N*-acetyl-D-glucosamine head group) and only the comparison of their intensities will indicate the presence of the immunoglobulin in the mixed monolayer provided that the transferred amount of glycolipids was the same in all the cases. The integration of the CH stretching vibrations, previously correlated to the thickness of trans-

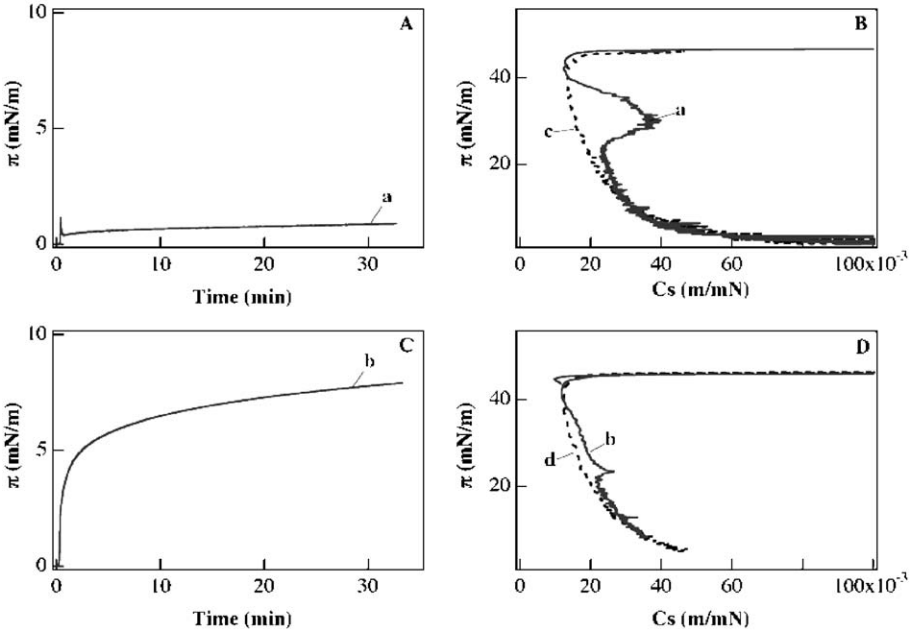


Fig. 6. Kinetics of the surface film formation (A, C) and two-dimensional compressibility curves (B, D) of the IgG–glycolipid monolayer formed at two VSD_{init}. (a) 16.8×10^7 vesicles/cm^2; (b) 33.8×10^7 vesicles/cm^2. Curves c and d correspond to the π–Cs curve of pure glycolipid monolayer, (c) 6.34×10^7 vesicles/cm^2; (d) 12.8×10^7 vesicles/cm^2 ($I = 1.17$).

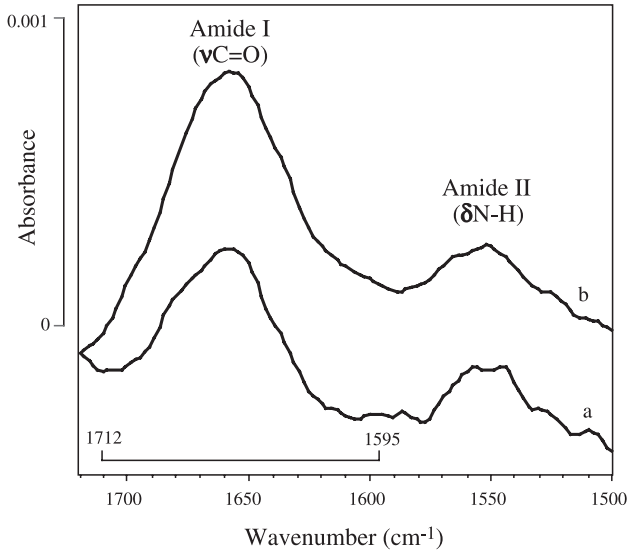


Fig. 7. A region (1750–1500 cm^{-1}) of typical a ATR-FTIR spectra of pure (a) and IgG–glycolipid (b) monolayer formed by vesicle spreading under a low-ionic-strength phosphate buffer ($I=0.023$) and transferred onto a germanium plate.

ferred layers [51], allowed us to determine that, whatever the ionic strength (from 0.023 to 0.23) and the presence or absence of immunoglobulin, it was equal to 0.2 au. This value, constant for all the experimental conditions, is reflecting the thickness of one transferred monolayer, in agreement with our previous results [13]. Due to their molar ratio, the contribution of the CH stretching vibrations of the protein was supposed negligible with regard to that of the glycolipid. The quantitative spectrum analysis of glycolipid and IgG-glycolipid monolayers is presented in Table 1 (ionic strengths varying from 0.023 to 0.23). For both kinds of monolayers, the integration value of the Amide I band presents an unexplained minimum value at the ionic strength of 0.12. However, the ratio of the Amide I integration values in the presence and in the absence of protein (column 3, Table 1) undoubtedly reflects the presence of the protein in the mixed monolayer. The immunoglobulin is still present in the condensed monolayer, even after transfer, and the retained amounts of the latter increases with the increase of ionic strength of the buffer.

Table 1
Quantitative spectrum analysis of Amide I band spectral regions of glycolipid and IgG–glycolipid monolayers after transfer onto germanium plates[a]

Subphase ionic strength	Integration value of Amide I band [b](au)		
	With IgG (a) (1712–1595 cm^{-1})	Without IgG (b) (1705–1610 cm^{-1})	Amide I ratio (a/b)
0.023	0.043	0.030	1.4
0.12	0.036	0.016	2.25
0.23	0.200	0.076	2.63

[a] At a surface pressure of 30 mN/m.
[b] Integrated area calculated by Nicolet's software.

Further insights into the phenomena responsible for the lateral compressibility variations (i.e. protein ejection or protein reorientation) were obtained by studying in detail a condensed monolayer presenting no significant modifications either in the π–A isotherm or in the lateral compressibility compared with the pure glycolipid film (Fig. 8). The interfacial film was transferred at 30 mN/m and the integration of the Amide I band displayed values of 0.031 and 0.061 au, for pure and mixed interfacial films, respectively. The integration ratio (1.8) indicates that the immunoglobulin was inserted in the condensed glycolipid film, even if no in-plane elasticity variation was recorded during the monolayer compression. Since at low ionic strength, the protein expulsion process could not be prevented, the lateral monolayer compressibility variation recorded during the mixed monolayer compression could be partly attributed to a protein reorientation process.

Therefore, the in-plane monolayer elasticity modification recorded when the spreading kinetics was mainly controlled by the diffusion step could not be ascribed to a total ejection of IgG from the mixed monolayer. The increase with the ionic strength of the Amide I band intensity obtained for the IgG–glycolipid interfacial film was in agreement with a higher amount of immunoglobulin retained in the glycolipidic matrix.

3.5. Immunoaffinity of IgG in the transferred monolayer

The immunoaffinity of the immunoglobulin inserted in the mixed monolayer was assessed through the acetylcholinesterase activity detected by the Ellman's method after enzyme immunoassociation. Table 2 presents the results obtained for monolayers formed under saturating or non-

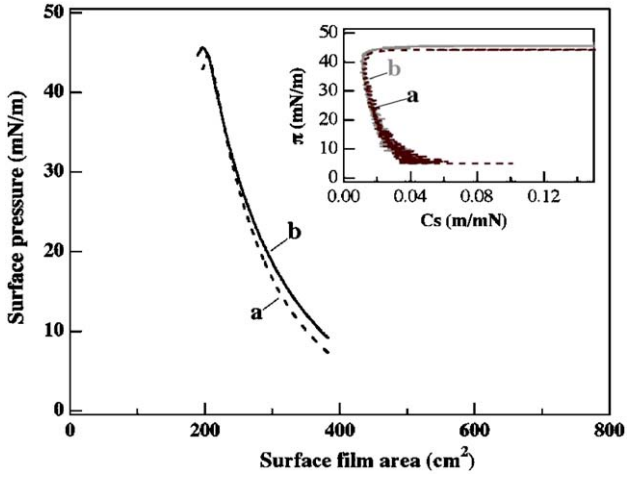


Fig. 8. π–A isotherm and π–Cs diagrams (inset) of pure glycolipid (a) and IgG–glycolipid (b) monolayer formed under saturating spreading conditions ($VSD_{init}=68.0\times10^{7}$ vesicles/cm^2) and at a low subphase ionic strength ($I=0.023$). No modifications were found on both π–A isotherm and π–Cs diagrams of the mixed monolayer compared with the pure glycolipid film.

Table 2
Acetylcholinesterase activity obtained after enzyme immunoassociation on transferred IgG–glycolipid monolayers[a]

VSD_{init} (vesicles/cm^2)	Transfer surface pressure (mN/m)	AChE activity (EU[b]/cm^2)
16.8×10^{7}[c]	30	0.552
	36	0.392
33.8×10^{7}[d]	30	0.255

[a] The monolayers were formed under subphase ionic strength of 0.82.
[b] EU: 1 Ellman's unit was defined as the enzyme activity producing an absorbance increase of 1 unit (412 nm) at 25 °C, in 1 min, in 1 ml of medium and for an optical path length of 1 cm.
[c] VSD_{init} for which in-plane elasticity modification was observed during compression (nonsaturating conditions).
[d] VSD_{init} for which no in-plane elasticity modification was observed during compression (saturating conditions).

saturating spreading conditions and transferred at different surface pressures. It is noteworthy that in the absence of immunoglobulin in the monolayer, no activity was detected, indicating that no unspecific adsorption occurred on the glycolipid monolayer (data not shown). For a monolayer formed in nonsaturating conditions ($VSD_{init} = 16.8 \times 10^7$ vesicles/cm^2), the activity was optimum when the monolayer was transferred at the maximal compressibility ($\pi_T = 30$ mN/m) and decreased when the monolayer was transferred at the end of the compressibility modification phase ($\pi_T = 36$ mN/m). This evolution could be ascribed either to the partial ejection of the immunoglobulin during the compression or to the effect of high surface pressures. Indeed, as previously reported for pure immunoglobulin monolayers, the immunoaffinity decreased with a surface pressure increase; in closely packed monolayers, the mobility of the Fab fragments that bind the antigen could be reduced [52]. In monolayers formed under saturating conditions ($VSD_{init} = 33.8 \times 10^7$ vesicles/cm^2), an activity can also be detected, even in the absence of in-plane elasticity modification during the compression. However, the activity measured was lower than that detected at 36 mN/m, for $VSD_{init} = 16.8 \times 10^7$ vesicles/cm^2, suggesting that under saturating spreading conditions a smaller amount of IgG is originally retained in the interfacial film. In such conditions, protein ejection could occur during the monolayer formation. Nevertheless, these results clearly demonstrate that the antibody was active in the monolayer even if no compressibility was recorded during the compression.

3.6. General discussion

As previously mentioned, a modification of the lateral monolayer compressibility of a mixed proteo-lipidic monolayer can be ascribed to an expulsion or a molecular reorientation of the protein. For pure antibody monolayers, it has been previously reported that the Y-shaped IgG, lying parallel to the interface at low surface pressures, gradually reorientates to a vertical position during the monolayer compression [53–56]. A similar molecular reorientation is assumed to be partly responsible for the in-plane elasticity modification observed for the IgG-glycolipid monolayers. Furthermore, the dependence of in-plane elasticity modifications on the spreading kinetics suggests that the change of orientation of IgG is likely the predominant process explaining the lateral compressibility variations. Indeed, if the lateral compressibility variations would be predominated by a protein expulsion mechanism, it should be at least as intense for the highest VSD_{init} (where the kinetics brings more material at the interface) as for the smallest ones. Moreover, even if the phenomenon decreases with the subphase ionic strength, it is still present. Therefore, the reorientation of the immunoglobulin in the glycolipid matrix appears to be prevailing during the compression of the mixed IgG–glycolipid monolayers, although protein expulsion cannot be excluded. The magnitude of this molecular reorientation process can be modified by the initial interface saturation, directly related to VSD_{init}. Fig. 9 presents a schematic illustration of the postulated steps occurring during the surface film formation from vesicle spreading. When no interfacial saturation is achieved (diffusion-controlled kinetics), IgG molecules can initially lie in the lipid environment with the Y-plane parallel to the air–buffer interface. During the compression, most of them can raise to a more vertical position (even if some are expelled), increasing the monolayer compressibility. When the interfacial saturation is achieved (transformation-controlled kinetics), glycolipid molecules prevents the proteins to lie horizontally at the air–liquid interface and constrains them to stand more vertically in the lipid matrix. Consequently, the immunoglobulin directly reaches a position close to the final orientation and reorientates less during compression.

The possibility for the immunoglobulin to stay included in the lipidic matrix, during both vesicle disintegration and monolayer compression, could be likely due to two parameters. On the one hand, weak carbohydrate/carbohydrate hydrophilic interactions could exist between the glycolipid heads and the glycan moiety of immunoglobulins, located in their hinge region. On the other hand, hydrophobic interactions could embed the hydrophobic Fc fragment of IgG in the lipid moiety of the glycolipid leaflets and induce a preferential orientation. This embedment could be favoured by the high fluidity of the hydrocarbon chains of the glycolipid allowing the conformational adaptation.

In the absence of in-plane elasticity modification, protein ejection from the interface could occur during the vesicles disintegration because of the rapid interfacial saturation by the glycolipid imposing a lateral surface pressure, before the monolayer compression. This could explain the lower enzyme activity retained under saturating conditions. Nevertheless, in saturating or nonsaturating conditions, the immunoglobulin seems correctly

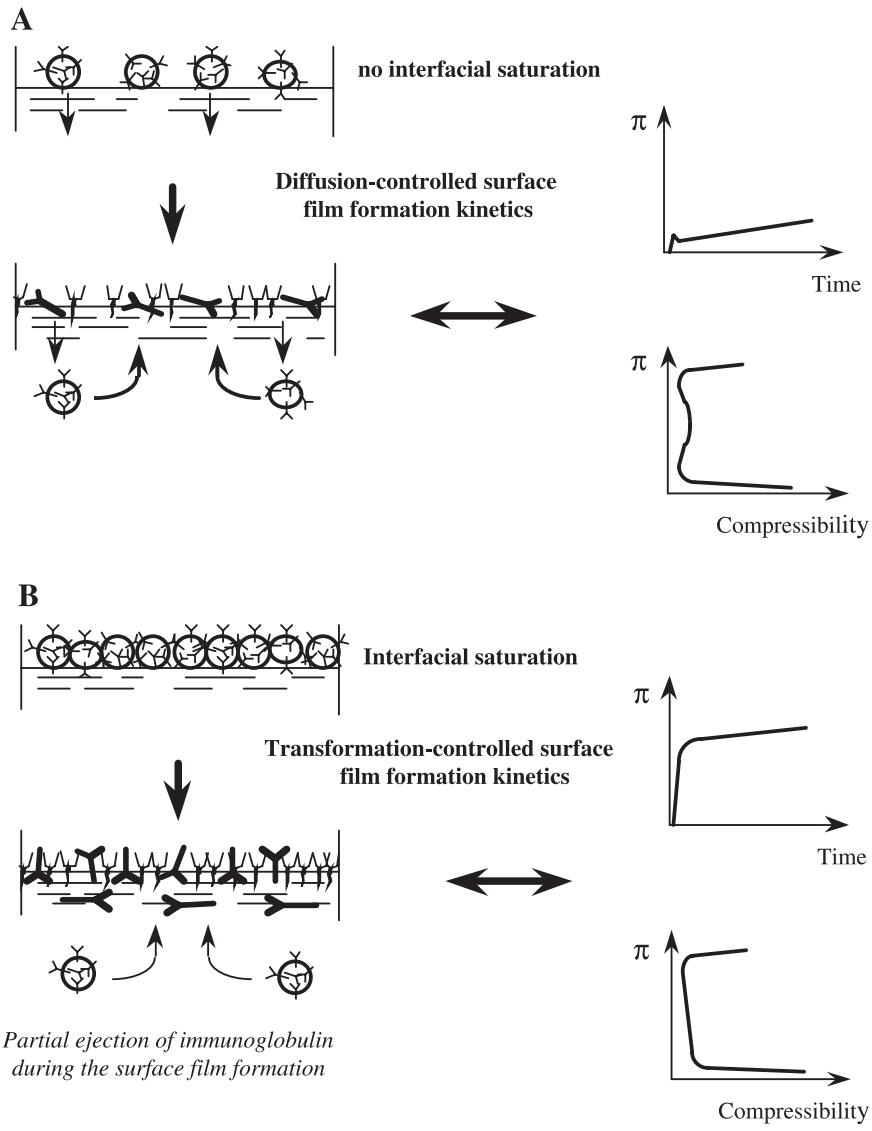


Fig. 9. Schematic representation of the possible steps occurring during the formation of IgG mixed glycolipid interfacial film from liposome spreading. (A) No interfacial saturation ($VSD_{init} < 15 \times 10^7$ vesicles/cm^2). (B) Interfacial saturation ($VSD_{init} > 30 \times 10^7$ vesicles/cm^2).

inserted and orientated in the condensed film to keep its immunoactivity.

4. Conclusion

This study focused on the influence of the kinetics of the surface film formation on the orientation of an antibody inserted in a mixed glycolipid monolayer formed by proteo-glycolipid vesicles spreading. The stability of the proteo-lipidic interactions, created during the vesicle assembling before the interfacial film formation, favours the insertion of a soluble protein in a lipid environment. The opportunity to detect a strong immunoaffinity after transfer of the mixed monolayer at a relative high surface pressure indicates that an antibody is embedded in a favourable orientation to express its activity. Thus, interfacial spreading of proteo-vesicle together with Langmuir–Blodgett technique appears as an efficient method to develop organised nanostructures. The combination of these methods based on the self-organisation ability of biomolecules allows both protein insertions in a predetermined orientation in the vesicle lipidic membranes and the enhancement of the proteo-lipidic organisation using lateral surface pressure. The stability of such molecular assemblies have been investigated before its integration in new biosensor concepts.

Acknowledgements

Thanks are due to Dr. J. GRASSI (CEA, Saclay, France) for his generous gift of the ascitic fluid and to Dr. C. BON (Institut Pasteur, Paris, France) for his generous gift of the

acetylcholinesterase of *Bungarus fasciatus* venom and for their interest in this work.

References

[1] T. Moriizumi, Langmuir–Blodgett films as chemical sensors, Thin Solid Films 160 (1988) 413–431.

[2] J.-I. Anzaï, K. Furuya, C.W. Chen, T. Osa, T. Matsuo, Enzyme sensors based on ion-sensitive field effect transistor. Use of Langmuir–Blodgett membrane as a support for immobilizing penicillinase, Anal. Sci. 3 (1987) 271–272;
J.-I. Anzaï, J.-Y. Hashimoto, T. Osa, T. Matsuo, Penicillin sensors based on an ion-sensitive field effect transistor coated with stearic acid Langmuir–Blodgett membrane, Anal. Sci. 4 (1988) 247–250;
J.-I. Anzaï, S. Lee, T. Osa, Enzyme-immobilized Langmuir–Blodgett membranes for biosensor application. Use of highly branched polyethyleneimine as a spacer for immobilizing α-chymotrypsin and urease, Makromol. Chem., Rapid Commun. 10 (1989) 167–170;
J.-I. Anzaï, T. Osa, Langmuir–Blodgett membranes in chemical sensor applications, Sel. Electrode Rev. 12 (1990) 3–34.

[3] M. Sriyudthsak, H. Yamagishi, T. Moriizumi, Enzyme-immobilized Langmuir–Blodgett film for a biosensor, Thin Solid Films 160 (1988) 463–469.

[4] H. Tsuzuki, T. Watanabe, Y. Okawa, S. Yoshida, S. Yano, K. Koumoto, M. Komiyama, Y. Nihei, A novel glucose sensor with a glucose oxidase monolayer immobilized by Langmuir–Blodgett technique, Chem. Lett. (1988) 1265–1268.

[5] Y. Okahata, T. Tsuruta, K. Ijiro, K. Ariga, Langmuir–Blodgett films of an enzyme–lipid complex for sensor membranes, Langmuir 4 (1988) 1373–1375;
Preparations of Langmuir–Blodgett films of enzyme–lipid complexes: a glucose sensor membrane, Thin Solid Films 180 (1989) 65–72.

[6] J.R. Li, M. Cai, T.F. Chen, L. Jiang, Enzyme electrodes with conductive polymer membranes and Langmuir–Blodgett films, Thin Solid Films 180 (1989) 205–210.

[7] S. Miyauchi, S. Arisawa, T. Arise, R. Yamamoto, Study of concentration of an enzyme immobilized by Langmuir–Blodgett films, Thin Solid Films 180 (1989) 293–298;
S. Arisawa, T. Arise, R. Yamamoto, Concentration of enzymes adsorbed onto Langmuir films and characteristics of a urea sensor, Thin Solid Films 209 (1992) 259–263;
S. Arisawa, R. Yamamoto, Quantitative characterization of enzymes adsorbed on to Langmuir–Blodgett films and the application to a urea sensor, Thin Solid Films 210/211 (1992) 443–445.

[8] T. Tatsuma, H. Tsuzuki, Y. Okawa, S. Yoshida, T. Watanabe, Bifunctional Langmuir–Blodgett film for enzyme immobilization and amperometric biosensor sensitization, Thin Solid Films 202 (1991) 145–150.

[9] S.Yu. Zaitsev, N.A. Kalabina, V.P. Zubov, Biosensors based on glucose oxidase Langmuir films, J. Anal. Chem. USSR 45 (1991) 1054–1056;
S.Yu. Zaitsev, Th. Hanke, U. Wollenberger, E. Ebert, N.A. Kalabina, V.P. Zubov, F. Scheller, Mono- and multilayer membranes with adsorbed glucose oxidase, Bioorg. Khim. 17 (1991) 767–772.

[10] Th. Hanke, U. Wollenberger, B. Ebert, F. Scheller, S.Yu. Zaitsev, Glucose oxidase/lipid mixed LB-Films on a Pt electrode application as sensor model, in: F. Scheller, R.D. Schmid (Eds.), Biosensors, Fundamentals, Technologies and Applications, GBF Monographs vol. 17, VCH Publishers, New York, 1992, pp. 43–46.

[11] C. Fiol, J.-M. Valleton, N. Delpire, G. Barbey, A. Barraud, A. Ruaudel-Texier, Elaboration of a glucose biosensor based on Langmuir–Blodgett technology, Thin Solid Films 210/211 (1992) 489–491.

[12] P. Pal, D. Nandi, T.N. Misra, Immobilization of alcohol dehydrogenase enzyme in a Langmuir–Blodgett film of stearic acid: its application as an ethanol sensor, Thin Solid Films 239 (1994) 138–143.

[13] A.P. Girard-Egrot, R.M. Morélis, P.R. Coulet, Bioactive nanostructure with glutamate dehydrogenase associated with LB films: protecting role of the enzyme molecules on the structural lipidic organization, Thin Solid Films 292 (1997) 282–289.

[14] A.P. Girard-Egrot, R.M. Morélis, P.R. Coulet, Direct bioelectrochemical monitoring of choline oxidase kinetic behaviour in Langmuir–Blodgett nanostructures, Bioelectrochem. Bioenerg. 46 (1998) 39–44.

[15] K. Wan, J.M. Chovelon, N. Jaffrezic-Renault, Enzyme-octadecylamine Langmuir–Blodgett membranes for ENFET biosensors, Talanta 52 (2000) 663–670.

[16] J.M. Chovelon, F. Gaillard, K. Wan, N. Jaffrezic-Renault, Influence of the surface pressure on the organization of mixed Langmuir–Blodgett films of octadecylamine and butyrylcholinesterase. 2. Film transferred onto silica support, Langmuir 16 (2000) 6228–6232.

[17] M. Yasuzawa, M. Hashimoto, S. Fujii, A. Kunugi, T. Nakaya, Preparation of glucose sensors using the Langmuir–Blodgett technique, Sens. Actuators, B 65 (2000) 241–243.

[18] B. Yang, H. Matsumura, K. Furusawa, The interactions between PC vesicles and the air/water and oil/water interfaces, Colloid Surf., A Physicochem. Eng. Asp. 148 (1999) 191–198.

[19] M. Gugliotti, H. Chaimovich, M.J. Politi, Fusion of vesicles with the air–water interface: the influence of polar head group, salt concentration, and vesicle size, Biochim. Biophys. Acta 1463 (2000) 301–306.

[20] M. Gugliotti, M.J. Politi, The role of the gel/liquid crystalline phase transition in the lung surfactant cycle, Biophys. Chem. 89 (2001) 243–251.

[21] R. Verger, F. Pattus, Spreading of membranes at the air–water interface, Chem. Phys. Lipids 16 (1976) 285–291;
F. Pattus, P. Desnuelle, R. Verger, Spreading of liposomes at the air/water interface, Biochim. Biophys. Acta 507 (1978) 62–70.

[22] M. Obladen, D. Popp, C. Schöll, H. Schwarz, F. Jähnig, Studies on lung surfactant replacement in respiratory distress syndrome rapid film formation from binary mixed liposomes, Biochim. Biophys. Acta 735 (1983) 215–224.

[23] T. Ivanova, G. Georgiev, I. Panaiotov, M. Ivanova, M.A. Launois-Surpas, J.E. Proust, F. Puisieux, Behavior of liposomes prepared from lung surfactant analogues and spread at the air–water interface, Prog. Colloid & Polym. Sci. 79 (1989) 24–32.

[24] M.A. Launois-Surpas, Tz. Ivanova, I. Panaiotov, J.E. Proust, F. Puisieux, G. Georgiev, Behavior of pure and mixed DPPC liposomes spread or adsorbed at the air–water interface, Colloid Polym. Sci. 270 (1992) 901–911.

[25] C.S. Vassilieff, I. Panaiotov, E.D. Manev, J.E. Proust, Tz. Ivanova, Kinetics of liposome disintegration from foam film studies: effect of the lipid bilayer phase state, Biophys. Chem. 58 (1996) 97–107.

[26] L. Marron-Brignone, R.M. Morélis, J.-P. Chauvet, P.R. Coulet, Insertion of luciferase in Langmuir–Blodgett films through enzyme–glycolipid vesicles, Langmuir 16 (2000) 498–503.

[27] F. Pizzolato, R.M. Morélis, P.R. Coulet, Enzymatic monolayers from proteolipidic Langmuir–Blodgett films for biosensors, Quim. Anal. 19 (2000) 32–37.

[28] A.P. Girard-Egrot, R.M. Morélis, P. Boullanger, P.R. Coulet, Immunological proteo-glycolipidic interfacial film obtained from spreading of liposomes including ascitic fluid, Colloids Surf., B Biointerfaces 18 (2000) 125–135.

[29] L. Dziri, B. Desbat, R.M. Leblanc, Polarization-modulated FT-IR spectroscopy studies of acetylcholinesterase secondary structure at the air–water interface, J. Am. Chem. Soc. 121 (1999) 9618–9625.

[30] S.V. Mello, C. Coutures, R.M. Leblanc, T.-C. Cheng, V.K. Rastogi, J.J. DeFrank, Interaction between hydrolase and paraoxon studied by surface chemistry in situ at air–water interface, Talanta 55 (2001) 881–887.

[31] A.P. Girard-Egrot, J.-P. Chauvet, P. Boullanger, P.R. Coulet, Glycolipid and monoclonal immunoglobulin–glycolipidic liposomes spread onto high ionic strength buffers: evidence for a true monolayer formation, Langmuir 17 (2001) 1200–1208.

[32] A.P. Girard-Egrot, J.-P. Chauvet, P. Boullanger, P.R. Coulet, IgG_1-

glycolipidic LB films obtained by vertical deposition of an interfacial film formed through proteo-liposome spreading at the air/water interface, Colloids Surf., B Biointerfaces 23 (2002) 319–325.
[33] S. Godoy, J.-P. Chauvet, P. Boullanger, L.J. Blum, A.P. Girard-Egrot, New functional proteo-glycolipidic molecular assembly for biocatalysis analysis of an immobilized enzyme in a biomimetic nanostructure, Langmuir 19 (2003) 5448–5456.
[34] P. Boullanger, M.R. Sancho-Camborieux, M.N. Bouchu, L. Marron-Brignone, R.M. Morélis, P.R. Coulet, Synthesis and interfacial behavior of three homologous glycero neoglycolipids with various chain lengths, Chem. Phys. Lipids 90 (1997) 63–74.
[35] F. Behroozi, Theory of elasticity in two dimensions and its application to Langmuir–Blodgett films, Langmuir 12 (1996) 2289–2291.
[36] J.T. Davies, E.K. Rideal, Interfacial Phenomena, Academic Press, New York, 1963, p. 265.
[37] M. Subirade, C. Salesse, D. Marion, M. Pézolet, Interaction of a nonspecific wheat lipid transfer protein with phospholipid monolayers imaged by fluorescence microscopy and studied by infrared spectroscopy, Biophys. J. 69 (1995) 974–988.
[38] M. Léonard, R.M. Morélis, P.R. Coulet, Linked influence of pH and cations on fatty acid monolayer integrity related to high-quality Langmuir–Blodgett films, Thin Solid Films 260 (1995) 227–231.
[39] L. Marron-Brignone, R.M. Morélis, P.R. Coulet, Synthetic glycolipids monolayer and related Langmuir–Blodgett films: importance of the amphipathic balance, J. Colloid Interface Sci. 191 (1997) 349–356.
[40] G.L. Ellman, K.D. Courtney, V. Andres Jr., R.M., Featherstone, Determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95.
[41] H. Schindler, Exchange and interactions between lipid layers at the surface of a liposome solution, Biochim. Biophys. Acta 55 (1979) 316–336.
[42] Tz. Ivanova, V. Raneva, I. Panaiotov, R. Verger, Kinetics of spreading of DOPC liposomes at the air–water interface subjected to phospholipase A_2 hydrolisis, Colloid Polym. Sci. 271 (1993) 290–297.
[43] I. Panaiotov, Tz. Ivanova, K. Balashev, J. Proust, Spreading kinetics of dimyristoylphosphatidylcholine liposomes at the air/water interface below and above the main phase-transition, Colloid Surf., A Physicochem. Eng. Asp. 102 (1995) 159–165.
[44] I. Vikholm, J. Peltonen, O. Teleman, Atomic force microscope images of lipid layers spread from vesicle suspensions, Biochim. Biophys. Acta 1233 (1995) 111–117.
[45] I. Panaiotov, J.E. Proust, V. Raneva, Tz. Ivanova, Kinetics of spreading at the air–water interface of dioleoylphosphatidylcholine liposomes influenced by photodynamic lipid peroxidation, Thin Solid Films 244 (1994) 845–851.
[46] J.M. Smaby, V.S. Kulkarni, M. Momsen, R.E. Brown, The interfacial elastic packing interactions of galactosylceramides, sphingomyelins, and phosphatidylcholines, Biophys. J. 70 (1996) 868–877.
[47] Z.-W. Yu, J. Jin, Y. Cao, Characterization of the Liquid-Expanded to liquid–condensed phase transition of monolayers by means of compressibility, Langmuir 18 (2002) 4530–4531.
[48] P. Ihalainen, J. Peltonen, Miscibility of lipids in monolayers investigated through adsorption studies of antibodies, Langmuir 19 (2003) 2226–2230.
[49] J.M. Smaby, M.M. Momsen, H.L. Brockman, R.E. Brown, Phosphatidylcholine acyl unsaturation modulates the decrease in interfacial elasticity induced by cholesterol, Biophys. J. 73 (1997) 1492–1505.
[50] S.L. Keller, Miscibility transitions and lateral compressibility in liquid phases of lipid monolayers, Langmuir 19 (2003) 1451–1456.
[51] F. Kimura, J. Umemura, T. Takenaka, FTIR-ATR studies on Langmuir–Blodgett films of stearic acid with 1–9 monolayers, Langmuir 2 (1986) 96–101.
[52] A. Tronin, T. Dubrovsky, C. De Nitti, A. Gussoni, V. Erokhin, C. Nicolini, Langmuir–Blodgett films of immunoglobulines IgG. Ellipsometric study of the deposition process and immunological activity, Thin Solid Films 238 (1994) 127–132.
[53] A. Ahluwalia, D. De Rossi, A. Schirone, Antigen recognition properties of antibody monolayers, Thin Solid Films 210/211 (1992) 726–729.
[54] T.B. Dubrovsky, M.V. Demcheva, A.P. Savitsky, E.Yu. Mantrova, A.I. Yaropolov, V.V. Savransky, L.V. Belovolova, Fluorescent and phosphorescent study of Langmuir–Blodgett antibody films for application to immunosensors, Biosens. Bioelectron. 8 (1993) 377–385.
[55] A. Tronin, T. Dubrovsky, C. Nicolini, Comparative study of Langmuir monolayers of immunoglobulines G formed at the air–water interface and covalently immobilized on solid supports, Langmuir 11 (1995) 385–389.
[56] T. Dubrovsky, A. Tronin, C. Nicolini, Determination of orientation of the IgG molecules in immobilized Langmuir monolayers by means of binding with fragment specific anti-immunoglobulin antibodies, Thins Solid Films 257 (1995) 130–133.