2
Langmuir-Blodgett Technique for Synthesis of Biomimetic Lipid Membranes

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Abbreviations:
A: Area per molecule
AFM: Atomic Force Microscopy
DLPE: Dilauroylphosphatidylethanolamine
DMPC: Dimyristoylphosphatidylcholine
DMPE: Dimyristoylphosphatidylethanolamine
DOPC: Dioleoylphosphatidylcholine
DOPE: Dioleoylphosphatidylethanolamine
DPG: diposphatidylglycerol
DPPA: Dipalmitoylphosphatidic acid
DPPC: Dipalmitoylphosphatidylcholine
DPPE: Dipalmitoylphosphatidylethanolamine
DPPS: Dipalmitoylphosphatidylserine
DSPC: Distearoylphosphatidylcholine
DSPE: Distearoylphosphatidylethanolamine
ESP: Equilibrium Spreading Pressure
FSB: Free Supported Bilayer
GM1: Monosialoganglioside-GM1
IgG: Immunoglobulin G
incl.: included
LB: Langmuir-Blodgett
LC: Liquid Condensed
LE: Liquid Expanded
LS: Langmuir-Schaefer
MGDG: Monogalactosyldiglyceride
DGDG: Digalactosyldiglyceride
MLV: Multilamellar Vesicle
OTS: Octadecyltrichlorosilane
PC: Phosphatidylcholine
PE: Phosphatidylethanolamine
PG: Phosphatidylglycerol
PI: Phosphatidylinositol
PS: Phosphatidylserine
R_D: Monolayer deposition Rate
SAM: Self-Assembled Monolayer
SFA: Surface Force Apparatus
π: Surface pressure
γ: Surface tension
SPM: Palmitoyl-Sphingomyeline

2.1. Introduction

One growing aspect of nanotechnology concerns the controlled elaboration of nanoscale systems. Generally speaking, nanobiotechnology requires the organization of atoms and molecules in a two- or three-dimensional space. The efficiency of the nanofabrication strategy and the recent development of methods allowing a direct characterisation at the molecular scale open a new way in the development of self-organized nanostructures. The analysis at the

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molecular and supramolecular level of biological systems, like cell membranes, constitutes an outstanding model to devise "intelligent nanostructures" based on the self-molecular assembly of biological macromolecules. In this sense, biomimetic membranes, that can be self-assembled into lipid bilayers, provides the basic support structure for many applications in nanobiotechnology.

The concept of using biomolecules as an elementary structure to develop self-assembled superstructures of defined geometry has thus received considerable attention. In this way, the self-assembly ability of amphiphilic biomolecules such as lipids, to spontaneously organize into nanostructures mimicking the living cell membranes, appears as a suitable concept for the development of biomimetic membrane models. The potential of two-dimensional molecular self-assemblies is clearly illustrated by Langmuir monolayers of lipid molecules, which have been extensively used as models to understand the role and the organization of biological membranes[189] and to acquire knowledge about the molecular recognition process[44,67,82,165,190]. Langmuir-Blodgett (LB) technology allows building up lamellar lipid stacking by transferring a monomolecular film formed at an air/water interface – named *Langmuir monolayer* or *Langmuir film* – onto a solid support. When all parameters are optimized, this technique corresponds to one of the most promising for preparing thin films of amphiphilic molecules as it enables (i) an accurate control of the thickness and of the molecular organization, (ii) an homogeneous deposition of the monolayer over large areas compared to the dimension of the molecules, (iii) the possibility to transfer monolayers on almost any kind of solid substrate and (iv) to elaborate bilayer structures with varying layer compositions. Based on the self-assembled properties of amphiphilic biomolecules at the air/water interface, LB technology offers the possibility to prepare biomimetic layers suitable for immobilisation of bio-active molecules.

The development of biomimetic and functional proteo-lipidic nanostructures based on Langmuir-Blodgett technology and corresponding to highly organized molecular assemblies associating oriented biological compounds is of great interest in the nanobiotechnology field for many reasons. These reasons include, (i) used in contact with a chemical (or physical) device handling as a signal transducer, such molecular structures should open a new way in the biocatalysis investigations at a nanometric scale in a biomimetic situation. Indeed, the miniaturisation of the molecular recognition system must allow an analysis of organized biomimetic systems associating a biosensitive element; (ii) associated to microelectronic and optoelectronic devices, they should lead to the design of new bioelectronic hybrids and the development of novel nanobiosensors; (iii) deposited on an ovoid scaffold and integrating ion channels or pore proteins, they should be implied in the drug vectorisation and drug delivery.

This chapter will be concerned with the recent progress in the development of organized lipid bilayers based on the Langmuir-Blodgett technology. It focuses on the fundamental principles and practical aspects of the elaboration of biomimetic lipid bilayers through LB deposition. A brief overview on the different ways to incorporate biomolecules into LB membranes will also be presented.

2.2. Langmuir Monolayer Formation

The Langmuir-Blodgett technology is based on the particular properties of organic molecules like lipids, phospholipids or glycolipids to orient themselves at an air/water interface between the gaseous and the liquid phase to minimise their free energy and form an *insoluble monolyer* called *Langmuir film* (Figure 2.1A). The classical materials forming Langmuir monolayers are insoluble amphiphiles, composed of two distinct molecular regions: a hydrophilic ("water loving") headgroup which is easily soluble in water, and a hydrophobic ("water-hating") tail which is soluble in nonpolar solvents. When drops of a dilute solution of an amphiphilic molecule in volatile and water-immiscible solvent such as chloroform are applied to a pure water surface, they rapidly spread over the interface to cover all the available area. After solvent evaporation, the interfacial film results in a monomolecular layer of one-molecule thick, with the headgroups immersed in the water and the tailgroups remaining outside (pointing towards the gas phase). This specific orientation is dictated by the amphiphilic nature of the molecules; also named *surfactants* for *surface active*, since they are located

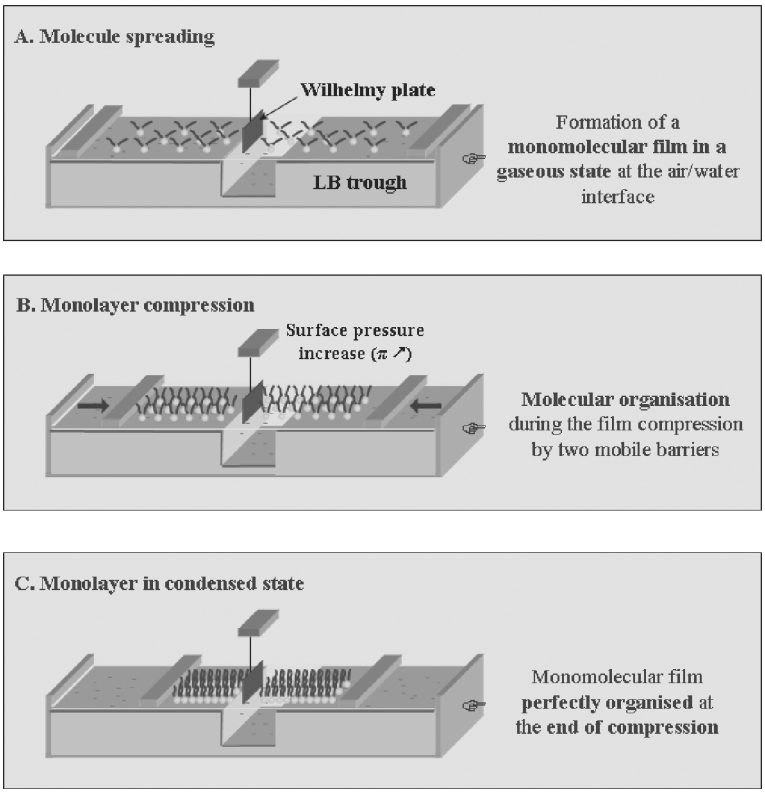

FIGURE 2.1. Langmuir monolayer formation.

at the air/water interface. The Langmuir monolayer represents an extreme case when considering adsorption to interfaces, because all the spread molecules are concentrated in a monomolecular interfacial film.

2.2.1. Surface Tension

The formation of an amphiphile monolayer is related to the particular thermodynamic properties of the air/liquid interface. The surface of a liquid has excess free energy due to the difference in environment between the surface molecules and those in the bulk. In the liquid, the molecules have a certain degree of attraction to each other. The degree of this attraction, also called *cohesion*, is dependent on the properties of the substance. In water, hydrogen bonding forces tend to set up loosely defined networks and the molecular interactions in the bulk are balanced by an equally attractive force in all directions (Figure 2.2). At the interface, a molecule is surrounded by fewer molecules than one in the bulk liquid and the equilibrium of forces is disrupted. The surface molecules experience an imbalance of forces due to unbalanced molecular attraction and a molecule at the air/water has a larger attraction towards the liquid than the gas phase. A net attractive force towards the bulk thus results from this situation and an air/water interface will spontaneously minimize its area and contract. Under this condition, the work done for extending a liquid surface is against this attractive force and consequently produces an increase of the free energy of the system. For an interface to be in equilibrium, as many molecules must leave the surface for the bulk of the liquid per time unit as diffuse from the bulk to the interface. However, due to the attractive force, more molecules will diffuse initially from the surface, thus increasing the mean atomic separation and therefore the intermolecular force between the surface molecules (Figure 2.2). The activation energy for a surface molecule escaping into the bulk will then increase until it is equal to that for molecules diffusing from the bulk to the surface, and a state of equilibrium is achieved[145]. The linear force acting on the surface molecules is the *surface tension* (γ). The thermodynamics of liquid surfaces has been initially reviewed by Birdi[19] and then by Gaines[53], and the surface tension of a plane interface can be expressed by the partial derivatives of free energy functions of the system with respect to the surface area S, as in equation 1.

$$\gamma = (\delta F/\delta S)_{T,V,ni} = (\delta G/\delta S)_{T,P,ni} \quad (1)$$

In equation 1, F and G are the Helmholtz or the Gibbs free energies of the system respectively, while temperature T, volume V, pressure P and amounts of all components n_i are held constant.

In the case of a *pure* liquid which is in equilibrium with its saturated vapour at the plane interface, the surface tension is also equal to the excess Helmholtz free energy per unit area, as in equation 2.

$$\gamma = F^s/A \quad (2)$$

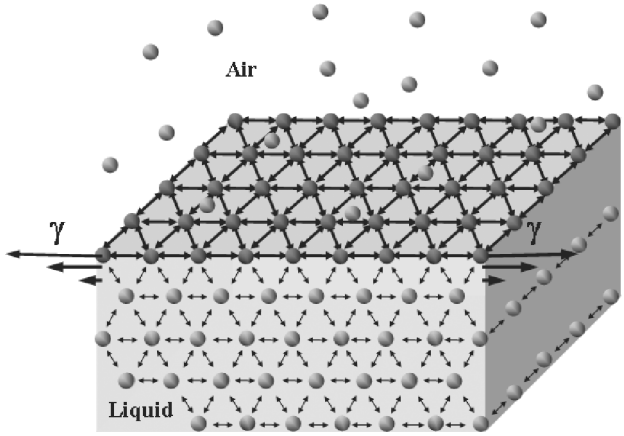

FIGURE 2.2. Surface tension at the air-water interface.

In equation 2, F^s refers to the surface excess free energy.

The common unit for surface tension is mM/m, since energy is usually expressed in [J] = [N.m] and surface area in [m^2]. Therefore, the surface tension can also be defined in term of a force per length unit representative of the cohesive energy present at an interface. It should be noticed here that the surface tension remains constant at a constant temperature but decreases with increasing temperature.

Polar liquids, such as water, have strong intermolecular interactions and thus, high surface tension. The surface tension of water is 72.8 mN/m at 20˚C and atmospheric pressure. This is an exceptionally high value compared to most other liquids and consequently makes water as a pre-eminent subphase for monolayer studies.

2.2.2. *Surfactants*

All the amphiphilic molecules are potentially surface active agents and substantially monolayer-forming materials. The reader may find a discussion on the range of a large variety of amphiphile compounds able (or previously used) to form insoluble monomolecular films in different reference books[53,145,158]. With the aim to synthesise biomimetic membranes, the most important types of amphiphilic molecules are *fatty acids*, *phospholipids* and *glycolipids*. *Cholesterol*, a type of steroid extremely abundant in the cell membrane, can also form insoluble monolayers but it is generally more studied mixed with other phospholipids[24,94,97,122,134,149,150,196,197,198] due to its implication in the formation of lipid microdomains or "*rafts*" in the cell membrane[2,26,168,169,170]. Figure 2.3 shows the principal structures of these different types of lipids and Table 2.1 gives the lipid composition of some characteristic biological membranes.

The amphiphilic nature of biological surfactants is responsible for their accumulation at the air/water interface. Their affinity for the air/water interface is

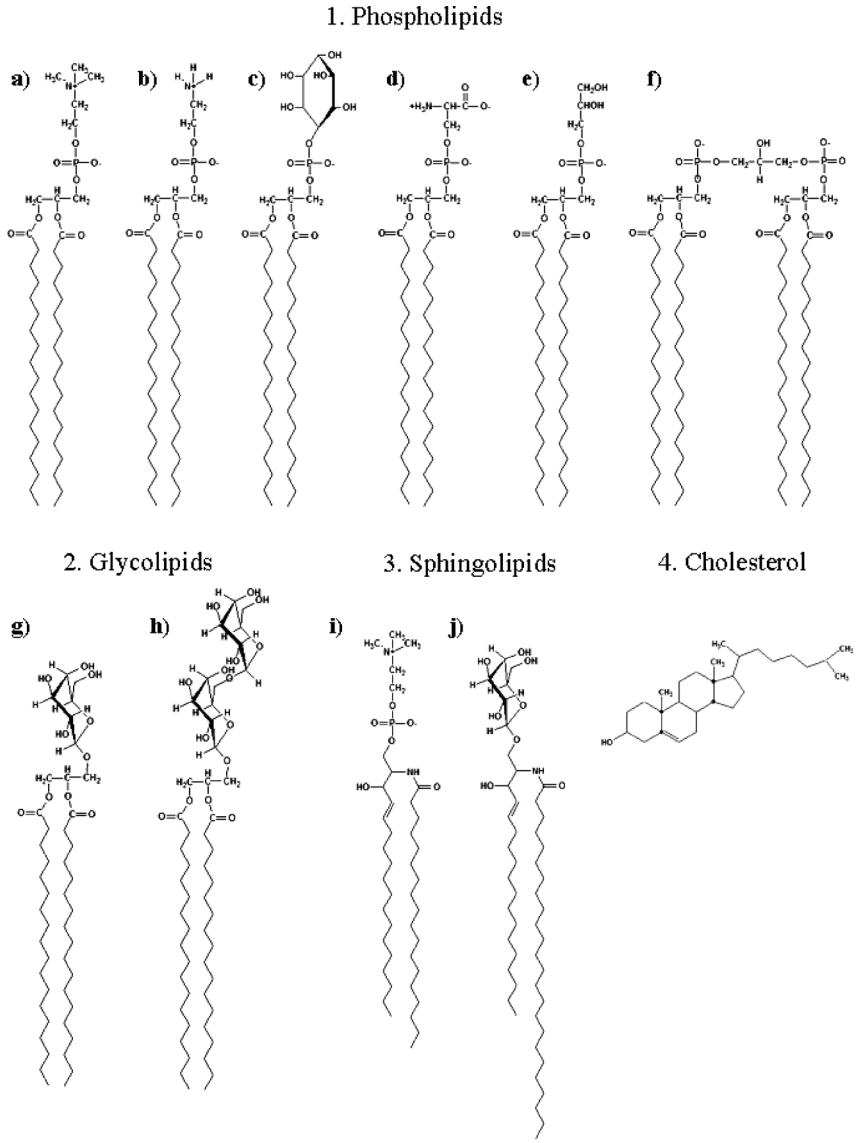

FIGURE 2.3. Structure of the main membrane lipids. 1) Phospholipids: a, phophatidylcholine (PC); b, phophatidylethanolamine (PE); c, phophatidylinositol (PI); d, phophatidylserine (PS); e, phophatidylglycerol (PG); f, diphophatidylglycerol (DPG). 2) Glycolipids: g, monogalactosyldiglyceride (MGDG); h, digalactosyldiglyceride (DGDG). 3) Sphingolipids: i, sphingomyeline; j, galactosylceramide. 4) Cholesterol. Membrane lipids are characterized by two hydrocarbon chains per molecule (not necessarily of the same length). In the most common lipids, the fatty acid alkyl chains are usually unbranched and one of the two is often unsaturated. The double chains are essential to the correct solid geometry that allows lipids to form membranes while the unsaturated chain helps to maintain the lipid fluidity. All phospholipids have a negative charge on

TABLE 2.1. Lipid composition of some characteristic biological membranes.

	Myelin	Erythrocyte	Mitochondria	*E. coli*	Chloroplast
Lipid/Protein (w/w)	3:1	1:3	1:3	1:3	1:1
Phospholipid*	32	56	95	100	12
incl. PC	11	23	48		
PE	14	20	28	80	
PI		2	8		
PS	7	11			
PG				15	12
DPG			11	5	
Glycolipid*					80
incl. MGDG					41
DGDG					23
Sphingolipid*	40	18			
Sterol*	25	25	5		

* Composition given in % of total lipid amount.
Phospholipids: PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PI: Phosphatidylinositol, PS: Phosphatidylserine, PG: Phosphatidylglycerol, DPG: diposphatidylglycerol.
Glycolipids: MGDG: monogalactosyl diglyceride, DGDG: digalactosyl diglyceride.

determined by the physico-chemical properties of the hydrophilic and hydrophobic parts. The hydrophilic part confers water solubility while the hydrophobic part (most often hydrocarbon chains) prevents water solubility. The monolayer-forming abilities of the amphiphiles is dependent on the balance between these two opposite forces, which are determined by the size of the hydrophobic tailgroup (*i.e.* the alkyl chain length) and the strength of the hydrophilic headgroup (*i.e.* its size, its polarity, its charge and its hydration capacity). If the hydrocarbon chains are too short, or the polar group too strong, the material would simply "dissolve" in the subphase and could not form a stable monolayer.

For an amphiphile formed by only one alkyl chain, like the *long-chain fatty acids*, the hydrocarbon chain has to be long enough to form an insoluble monolayer (generally more than 12 hydrocarbon in the chain, $(CH_2)_n$, n>12), since the first one or two methylene groups are solubilised in the water. If the chain is shorter, these compounds are too "soluble" in water and the molecules spread on the water surface tend to form micelles above their critical micellar concentration. This formation of micelles, corresponding to water soluble entities, prevents the formation of a stable monolayer at the air/water interface. Conversely, if the hydrophobic part is dominant (chain length too long for

FIGURE 2.3. *(Continued)* the phosphate group at pH 7.0. The polar groups of phosphatidylcholine PC (a) and phosphatidylethanolamine PE (b) contain both positive and negative charges and are zwitterionic. The polar groups of phosphatidylinositol PI (c), phosphatidylserine PS (d), phosphatidylglycerol PG (e), contain a negative charge and are anionic. Glyco- (g, h) and sphingolipids (I, j) are neutral lipids (Petty 1996).

instance), these amphiphiles tend to crystallize on the water surface and they do not form a monolayer at the interface. In this case, a phase separation between the water and the lipid solid phase occurs. It is difficult to determine the optimal length for the hydrocarbon chain because the film-forming ability is also dependent on the polar part of the amphiphile.

Most of the lipidic cell membrane components are composed of a negatively charged or *zwitterionic* headgroup at pH 7.0 (*phospholipids*) or contain a highly hydrophilic polar group (*glycolipids*), and a hydrophobic part which is constituted by two hydrocarbon chains per molecule and drastically reduces the water solubility of the complete lipidic membrane molecule. Typically, their solubility in the form of monomers is between 10^{-12}– 10^{-10} M. Consequently, many components of cell membranes form insoluble monolayers at the air/water interface since the lipid concentration in the aqueous subphase is negligible, and some of them may be built up into multilayer films by Langmuir-Blodgett deposition (described in section 2.3).

2.2.3. Surface Pressure

As earlier mentioned, the air/liquid interface possesses an excess free energy originating from the difference in environment between the surface molecules and those in the bulk. The spontaneous formation of a monolayer when an amphiphilic surfactant is placed on a liquid surface will affect the surface tension. The surface tension can be viewed as a negative pressure due to the attractive interactions of the water molecules at the interface, which will be lowered by accumulation of the amphiphiles at the air/liquid interface. The presence of a monomolecular film on a liquid surface invariably results in a reduction of the free energy of the system due to the creation of interactions between the hydrophilic polar group and the water surface molecules, thus reducing the surface tension. The resulting effect of the reduction of the surface tension leads to an expansion of an air/water interface in the presence of surfactants.

When the area of surface available to the interfacial film is large and the amount of surfactants sufficiently low to limit the interactions between adjacent amphiphiles molecules, the monolayer has a minimal effect on the liquid surface tension. If the available surface area to the monolayer is reduced by a compression system comprised of mobile barriers (Figures 2.1B & 2.1C), the intermolecular distance decreases and the surface tension is lowered. The amphiphile molecules (mainly their hydrocarbon chains) start to interact and exert a repulsive effect on each other. The force exerted by the film per unit length, corresponding to a two-dimensional analogue of a pressure, is called *surface pressure* (π). It is equal to the reduction of the pure liquid surface tension by the presence of the interfacial film, as in equation 3.

$$\pi = \gamma_0 - \gamma \tag{3}$$

In equation 3, γ_0 is the surface tension of the pure liquid and γ is the surface tension of the film-covered surface. It results from this equality that the maximum surface pressure for a monolayer on water surface at 20°C is 72.8 mN/m, and normally encountered values are much lower. The formation of a monolayer at the air/water interface is usually monitored by recording the surface pressure (π) – area (A) isotherm diagram, which will be discussed in the next section. Two fundamentally different approaches can be used to measure the surface pressure in the interfacial film during the monolayer compression: the *Langmuir balance* and the *Wilhelmy plate*.

The *Langmuir balance* method corresponds to the differential measurement of the force acting on a movable float separating a clean portion of the water (or aqueous) surface from the surface covered with the monolayer (Figure 2.4). The amplitude of the force exerted by the film, leading to a discrete displacement of the float (expansion of the monolayer-covered surface), is directly measured by a conventional balance connected to the float. The displacement of the float is usually small (~10 μm) and conveniently measured using a displacement transducer[158]. In this system, the force F acting on the float to move it a distance of dx and to expand the monolayer-covered surface of dS_m (*i.e.* reduce the clean water surface of dS_0) is related to the surface pressure π by equation 4.

$$F dx = \gamma dS_m - \gamma_0 dS_0 = (\gamma_0 - \gamma) dS = \pi \cdot l dx \tag{4}$$

In equation 4, dS_m = - dS_0, and l is the width of the monolayer. Then, the surface pressure is obtained from equation 5.

$$\pi = F/l \tag{5}$$

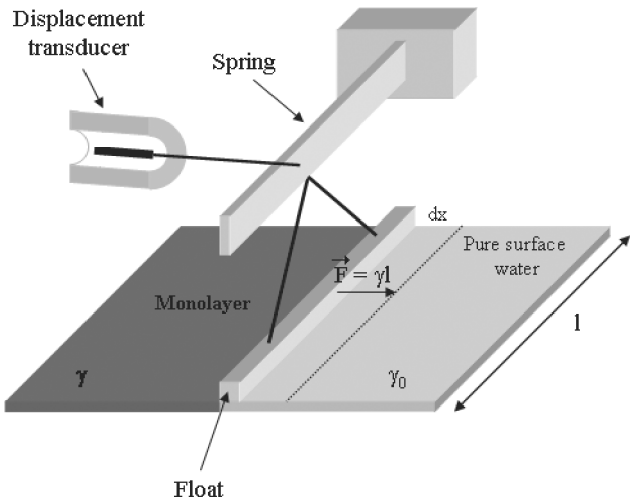

FIGURE 2.4. Principle of the Langmuir balance.

This technique was the first used by[101] to measure the surface pressure of a liquid covered by a thin film.

The *Wilhelmy plate* method is based on an absolute measurement of the force due to the surface tension on a plate, usually made of platinum or filter paper (ashless Whatman Chromatography paper Chr1), partially immersed in the subphase (Figure 2.5). The measurement is first performed on a clean surface and subsequently on the same surface covered by the monolayer. The variation due to the alteration in the surface tension is then converted into surface pressure with the help of the dimensions of the plate. Indeed, the forces acting on the plate consist of downward forces, such as gravity and surface tension, and upward force, such as the buoyancy due to the displacement of water. For a rectangular plate of dimensions L, w, and t, of material density ρ_p, immersed to a depth h in a liquid of density ρ_L, the net downward force, F_0, in the absence of a monolayer, is given by equation 6.

$$F_0 = \rho_p gLwt + 2\gamma_0(t+w)cos\theta_0 - \rho_L gtwh \tag{6}$$

In equation 6, g is the gravitational constant and θ_0 is the contact angle of the liquid on the solid plate[158].

With a monolayer-covered surface, the expression of the force, F_m, exerted on the plate is described by equation 7.

$$F_m = \rho_p gLwt + 2\gamma(t+w)cos\theta_m - \rho_L gtwh \tag{7}$$

In equation 7, θ_m is the contact angle of the liquid covered by the monolayer on the solid plate.

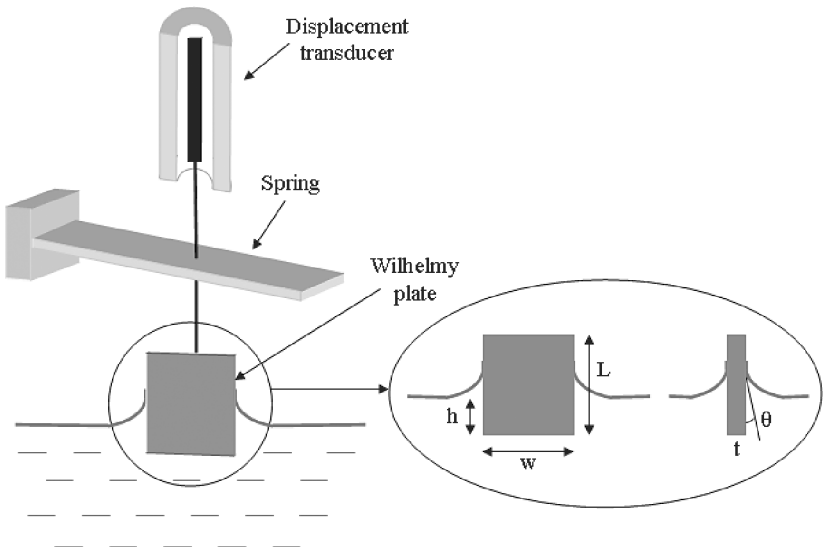

FIGURE 2.5. Principle of the Wilhelmy plate method.

The measurement of the change in the force exerted in the presence of the monolayer on a stationary plate (h maintained constant) is related to the change in surface tension by equation 8.

$$\Delta F = F_m - F_0 = 2(t+w)(\gamma cos\theta_m - \gamma_0 cos\theta_0) \tag{8}$$

If the plate is completely wetted by the liquid, the contact angles equal zero, and if it is thin enough so that t«w, then the change in force can be expressed as in equation 9.

$$\Delta F = 2w(\gamma - \gamma_0) = 2w\Delta\gamma \tag{9}$$

Thereby, the surface pressure, π, equal to the reduction of the pure liquid surface tension by the film, is related to the change in the force, ΔF, by equation 10.

$$\pi = -\Delta\gamma = -\Delta F/2w \tag{10}$$

Most of available commercial systems are now equipped with a Wilhelmy plate. The forces are generally measured with a sensitive electrobalance directly coupled to the plate. The sensitivity is around 10^{-3} mN/m. Alternatively, the force exerted vertically on the plate by the surface tension can be transformed into a small displacement by means of a spring, which is conveniently measured using a displacement transducer (Figure 2.5). One important drawback of the Wilhelmy method is the change in contact angle when the plate is covered with monolayer material. It appears from equations (8) and (9) that the surface pressure measurement requires a precise knowledge of the contact angle value. A zero contact angle value is ensured when a freshly cleaned plate is immersed in the clean liquid surface and becomes perfectly wetted. However, a change in contact angle occurs if monolayer material is deposited on the plate. This artefact may be obviated by using a fresh, clean and appropriate material for each experiment. Other experimental deviations may nevertheless appear, especially for very rigid monolayers, owing to the movement of the Wilhelmy plate[35].

2.2.4. *Surface Pressure (π) – Area (A) Isotherms*

The surface pressure (π) – Area (A) isotherm is a plot of the change in surface pressure as a function of the area available to each molecule on the aqueous subphase surface. This isotherm is the most common indicator of the monolayer formation and monolayer properties of an amphiphilic material. The isotherm is measured at constant temperature, usually under a pseudo-equilibrium condition, by continuously compressing the monolayer while monitoring the surface pressure. (Equilibrium values could be recorded by on a point-to-point compression process).

In a typical experiment, the amount of surfactants initially spread is sufficiently low so that the molecules are far enough apart on the water surface.

Under this condition, the molecules exert only small forces on one another and the resulting monolayer can be regarded as a *two-dimensional gas* due to the large distances between the molecules (Figure 2.1A). In the gaseous state, the monolayer has relatively little effect on the free energy of the aqueous subphase, even though the surfactant molecules have a natural tendency to aggregate. Therefore, the liquid surface tension remains unchanged and the surface pressure is very low (<1 mN/m). When the available area of the monolayer is reduced by a mobile compression barrier system the molecules become closer together, the intermolecular distance decreases and the surface tension diminishes. The hydrocarbon chains of the molecules begin to interact and the surface pressure start to increase (lift-off point). During the monolayer compression, the amphiphilic molecules self-organize and the monolayer will undergo several phase transformations analogous to the three-dimensional gaseous, liquid and solid states to finally form a floating monolayer perfectly ordered at the liquid surface. During this process, the hydrophilic and hydrophobic ends of the molecule ensure that the individual molecules are aligned in the same way (Figures 2.1B& 2.1C).

As the molecules forming Langmuir monolayers are insoluble amphiphiles, the total number of molecules unchanged during the whole compression. The a*rea per molecule*, which represents the mean area available to each molecule, is usually calculated by dividing the film area - determined by the barrier position during the compression - by the total number of molecules spread on the water surface. The *area per molecule*, A, is usually expressed in $Å^2$ or $nm^2.molecule^{-1}$. The continuous monitoring of the surface pressure as a function of the area per molecule gives rise to the surface pressure/area (π-A) isotherm diagram of the monolayer. Figure 2.6 depicts schematic π-A isotherms classically recorded for long-chain fatty acids (lipids with one alkyl chain) and phospholipids (natural membrane lipids presenting two alkyl chains). These diagrams are not meant to represent that observed for any particular substance, but shows most of the features obtained for these two classes of biological surfactants.

A number of distinct regions, named *phases*, can be distinguished on examining the isotherm. These phases are characteristic of different aggregation states that the molecules adopt in the monolayer during the compression and are identified as discontinuities in the isotherm. They correspond to various molecular organisations in which molecules have different degrees of freedom. They result from the molecular interaction forces occurring between the molecules in the floating film, and between the film and the subphase: the different monolayer states are both dependent on the van der Waals forces between the hydrocarbon chains, which are responsible for the cohesion within the film, and the magnitude of the attractive and repulsive forces existing between the headgroups. At a large area per molecule, the monolayer exists in the gaseous state (G, in Figure 2.6). The molecular interactions are small and there is no lateral adhesion in the interfacial film. The hydrophobic chains are distributed near the interface and present a large degree of freedom. This phase is generally encountered for a surface pressure lower than 0.5mN/m. As the gaseous phase is compressed, the hydrocarbon chains start to lift away and a first-order transition

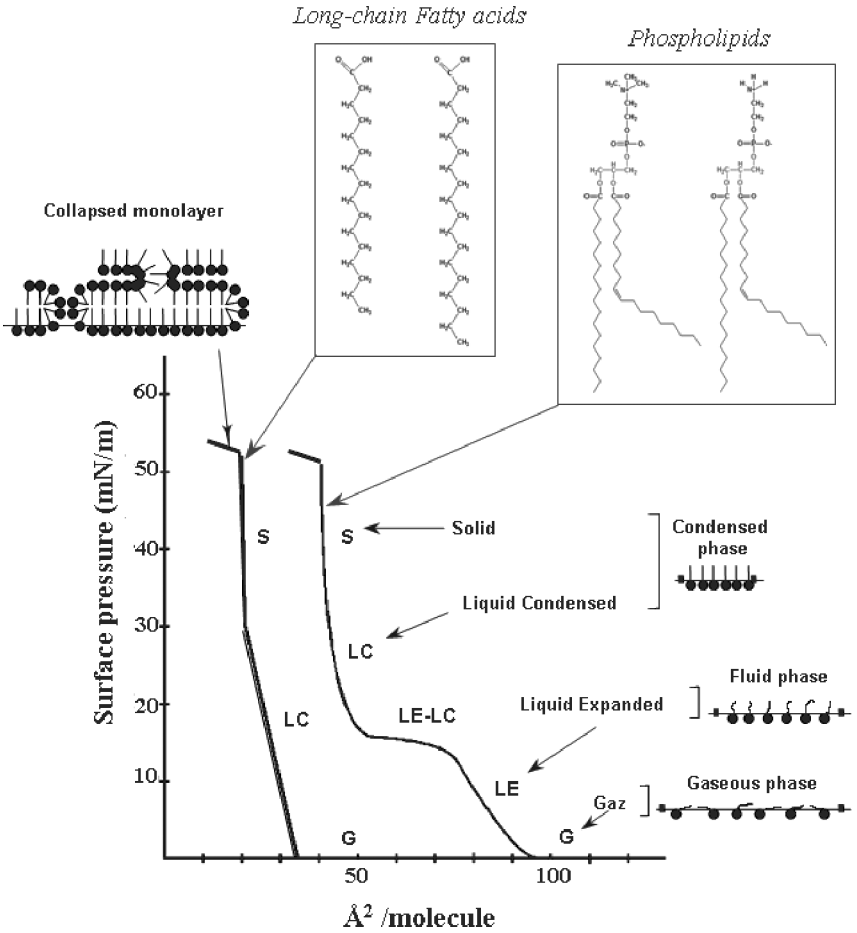

FIGURE 2.6. Schematic π-A isotherms of long-chain fatty acids or phospholipids. The overall shape of the isotherms mainly depends on the subphase temperature, the hydrocarbon chain length and the presence of unsaturated acyl chains.

from the "gas" to "liquid" state occurs. Usually, this is accompanied by a constant plateau region in the isotherm occurring at a surface pressure lower than 1mN/m owing to the weakness of interactions between the tailgroups. However, this portion of the isotherm is often not resolved by the Wilhelmy plate. As the surface area of the monolayer is further reduced, the liquid state, called "Liquid-Expanded" (LE) is formed. In the LE phase, the monolayer becomes coherent, but the molecules still possesses degrees of freedom. The hydrocarbon chains in such a film are randomly oriented and still present *gauche* conformations. In the expanded phase, the area per molecule varies considerably with the surface pressure and no relation between the observed molecular area and the dimensions of the constituent molecules is apparent: the area per molecule is larger than the area associated with the cross-section of cylindrical alkyl

chains (*i.e.* ≈0.19 nm^2 per unit). Under further compression, a second first-order thermodynamic transition from the liquid to the condensed states can occur in the monolayer. This Liquid Expanded - Liquid Condensed (LE-LC) transition phase is characterised by a second constant pressure region in the isotherm which occurs at area per molecule comprised between 0.6 and 0.8 $nm^2.molecule^{-1}$ for phospholipid monolayers. In the LE-LC phase transition, condensed lipid domains appear in the expanded phase. The coexistence of both condensed and expanded phases may be directly observed in the floating monolayer by Brewster angle microscopy[188] or by fluorescence microscopy after incorporation a small fluorescent dye probe into the film[98,114]. As the molecular area is progressively reduced, condensed phases (*i.e.* Liquid Condensed, LC, or Solid, S, state) may appear (Figure 2.6). The terms "condensed phases" included in fact different monolayer states similar to mesophases found in the smectic liquid crystals and presenting a well-defined *in-plane* structure[145], which can be characterised by X-ray diffraction[95]. In the condensed phases, the monolayer presents a strong lateral cohesion. The molecules are closely packed. The hydrocarbon chains are crystallized and uniformly oriented. The area per molecule is approaching that of the molecular cross-section (≈0.2$nm^2.molecule^{-1}$ for fatty acids and ≈0.4–0.5$nm^2.molecule^{-1}$ for phospho- and glycolipid molecules), thus confirming the interpretation of the condensed monolayers as a two-dimensional solid. The various states of the condensed monolayers are assumed to be related to different interactions and to different arrangements of the polar and hydrocarbon chain groups. For fatty acid monolayers, the transition between the different condensed phases are characterised by a decrease of tilt angle of the alkyl chains from the normal to the interface and for the highest surface pressures, the chain axes are vertically oriented. Finally, if compression is further applied to the monolayer, the phenomenon of *collapse* occurs at smaller surface areas. It is due to mechanical instability at very high surface pressures and molecules are forced out of the interfacial film. The monolayer loses its integrity. Molecular layers are riding on top of each other and disordered multilayers are being formed (Figure 2.6). The onset of collapse depends on many factors including the rate at which the monolayer is being compressed and the history of the film (ageing time).

The phase behaviour of the monolayer is mainly determined by the physical and chemical properties of the amphiphile. As shown in Figure 2.6, the liquid expanded state cannot exist and a direct transition from the gas to the condensed phase can take place. This behaviour is generally obtained for fatty acids with a long hydrocarbon chain, for which van der Waals forces between the hydrocarbon chains are largely responsible for the phase transitions. For the phospholipid or glycolipid molecules, which contain two hydrocarbon chains per molecule, the size of the hydrophilic headgroup influences the hydrocarbon chain packing, and consequently, the molecular aggregation state in the condensed monolayer. In a general manner, because of their polarity, size, shape, interaction with water and/or the neighbouring headgroups, many polar groups strongly influence the arrangement of the hydrocarbon chains and hence the characteristics of the π-A isotherm. It must be stressed here that the shape of the isotherm also depends

on the experimental conditions (*e.g.* temperature, pH, subphase composition), the hydrocarbon chain length, and the presence of unsaturated alkyl chains (disruption in the chain ordering). For instance, different transition phases can be observed when the subphase temperature varies. Reducing the temperature or lengthening the chain both enhance the intermolecular (chain-chain) interactions, tending to make the film more coherent and ordered (extension of the LC phase with a clear fading of the LE-LC transition). Hence, an increase (or a reduction) in the saturated chain length can, to some extend, be traded for a reduction (or an increase) in temperature [10,158].

In a general manner, π-A isotherms provide information on the monolayer stability at the air/water interface, the reorientation of the molecules in the two-dimensional system, and the existence of phase transitions and conformational transformations [182]. For detailed discussions on π-A isotherms, Readers can refer to different books and reviews dedicated to Langmuir and Langmuir-Blodgett films [43,53,145,158,182].

2.2.5. Monolayer Stability

The monolayer stability and hence, the monolayer homogeneity is one of a prerequisite to elaborate organized lipid Langmuir-Blodgett films with a high structural quality. Monolayer stability mainly depends on the monolayer dissolution into the subphase and on the mechanical stability in order to resist overcompressions.

In most cases, when a lipid monolayer is compressed, it is not in a perfect stable thermodynamic equilibrium. To be in a stable equilibrium, the monolayer should not be compressed at surface pressures exceeding the *equilibrium spreading pressure, ESP*. This equilibrium surface pressure is defined as that spontaneously generated when a crystalline sample of the solid material is placed in contact with a pure water surface[93]; the monolayer formed by molecules detaching from the crystal surface and spreading over the subphase is, thereby, in equilibrium with the crystals themselves. In other words, the *equilibrium spreading pressure* corresponds to the equilibrium pressure between the monolayer, the two-dimension state, and the crystal, the three-dimension state. This means that at any surface pressure higher than this, the monolayer has a tendency to aggregate into crystals by a nucleation and crystalline growth process. This process can be understood by comparison with the formation of an equilibrium vapour over a bulk solid. An equilibrium vapour exists for the solid in presence of its vapour. If this vapour is exceeded, *i.e.*, the vapour becomes *supersaturated*, molecule deposition onto the solid surface can occur. This should be also the case for the compressed monolayers which are expected to form crystals at surface pressures greater than *ESP* [145]. On a practical point of view, the equilibrium state between the monomolecular film and the solid crystal is fortunately most often approached very slowly, and the *ESP* is generally not attained in the course of an experiment. In many experiments, seemingly stable surface pressures up to higher values than *ESP* may be reached. The practical benefit of such a situation is the possibility

to handle interfacial films over a long period of time (if the surface pressure is not too high) without discernible loss of monolayer integrity (*e.g.* without slow collapse occurrence).

As already mentioned, the stability of the monolayer also depends on the solubility of the amphiphile monomers in the subphase. However, for insoluble amphiphilic molecules, a barrier to dissolution exists and the equilibrium with saturated subphase concentrations may be approached very slowly. Noticeably, compressed monolayers made of biological amphiphile molecules are stable over a long period of time without noticeable effect of the material dissolution (section 2.2.2).

Even if the processes responsible for the monolayer instability take long periods of time, it is important to consider that a floating monomolecular film is in a *metastable state* rather than in an absolutely stable equilibrium state and consequently, monolayer homogeneity (integrity) can be lost if the monomolecular film is not cautiously handled. Several factors can enhance the monolayer stability prior its transfer. These factors include the presence of multivalent ions in the aqueous phase as well as the subphase pH, which were found to play a critical role in determining the stability and the transferability of ionised monolayers. The influence of mono-, di- and trivalent ions has been then widely investigated[17,23,27,52,55,99,109,111,195] and even if it appears difficult to draw a universal phenomenon, some generalities can be highlighted. The interaction of divalent metal ions with the acid headgroup of fatty acids seems to depend on their electronegativity[41,151,164,203]. While metal ions with higher electronegativity interact covalently, those with lower electronegativity interact electrostatically. Such interactions affect the packing behaviour of the alkyl chains[164]. Complexes of metal ions with the acid headgroup of fatty acids generally causes the monolayer to be more condensed[103], and usually, more easily and more uniformly transferred. It can be noticed that the waiting times allowed for solvent evaporation prior to compression or after monolayer compression (*relaxation time*) can also influence the monolayer stability. In most cases, the monolayer is not completely stable after compression, but stabilises after some time. In a general manner, the monolayer stability can be checked either by measuring the decrease in surface pressure when the area is held constant, or by recording the decrease in film area when the surface pressure is kept constant[53]. Another way of checking monolayer stability corresponds to the dynamic cyclic π-A isotherms, also named *hysteresis experiments*. In such an experiment, the monolayer is successively compressed to a fixed surface pressure and then, relaxed to the original state. Some hysteresis phenomenon during the first compression/decompression cycle is normally observed, even for stable monolayers. It has been mainly ascribed to a difference in the *aggregation* (organisation of the molecules at the compression) and *relaxation* (disorganisation of the molecules at the decompression) processes, due to the 'non-return' of domains formed during the compression to their original state after decompression[177,92]. For poorly stable monolayers, a continuous shift of hysteresis loops towards lower mean molecular areas is observed in consecutive isotherms. This

can be attributed to a loss of film-forming molecules into the bulk (monolayer dissolution) or to molecular over-riding to form bi- and multilayer structures (monolayer collapse)[43]. As will see below, the stability of the floating monolayer is a crucial parameter to obtain high quality Langmuir-Blodgett films. Depending on the material being investigated, repeated compressions and expansions may be necessary to achieve a reproducible isotherm trace and produce a stable monolayer.

2.3. Langmuir-Blodgett Technique

When the surface pressure is sufficiently high to ensure lateral cohesion in the interfacial film, the floating monolayer can be transferred, like a carpet, from the water surface onto a solid plate "substrate". There are a number of different ways in which the monolayer may be transferred. This section is concerned exclusively with the universally known Langmuir-Blodgett technique[20,21], involving the vertical movement of a solid substrate through the monolayer/air interface.

2.3.1. Vertical Film Deposition Principles

Depending on the nature of the substrate, the first monolayer will be transferred as the substrate is respectively raised (*immersion*, or downstroke) or lowered (*emersion*, or upstroke) through the interfacial film (Figure 2.7). The monolayer is usually laid down during emersion of a hydrophilic substrate when the hydrophilic headgroups interact with the surface. If the substrate surface is hydrophobic, the monolayer will be transferred during immersion when the hydrophobic alkyl chains interact with it. For a hydrophilic surface, the substrate may be immersed into the subphase before the monolayer formation. After transfer of the first monolayer, a hydrophilic substrate becomes hydrophobic and a second monolayer will be deposited at the immersion. Conversely, a hydrophobic substrate becomes hydrophilic and the second monolayer will be transferred during the emersion. Subsequently, multilayers will build up by successive depositions of single layers on each traversal of the surface (*i.e.* the monolayer/air interface). Such a deposition mode called *Y-type* (Figure 2.7b) leads to a stack in head-to-head and tail-to-tail configuration, which is the most representative of the natural lamellar stacking of the biological membrane. Although this is the most frequently encountered situation, instances in which the floating monolayer is only transferred at the immersion (*X-Type* deposition, Figure 2.7a) or the emersion (*Z-type* deposition, Figure 2.7c) of the substrate have been reported. The type of deposition is mainly determined by the amphiphilic balance of the molecules and the nature of substrate (whether it is hydrophilic or hydrophobic) but it also depends on the dipping conditions (section 2.3.2). Surface pressure, deposition speed, pH, temperature and composition of the subphase, may affect the deposition mode[20,51,53,146]. Furthermore, mixed deposition types (*XY-type*) are sometimes encountered and the deposition

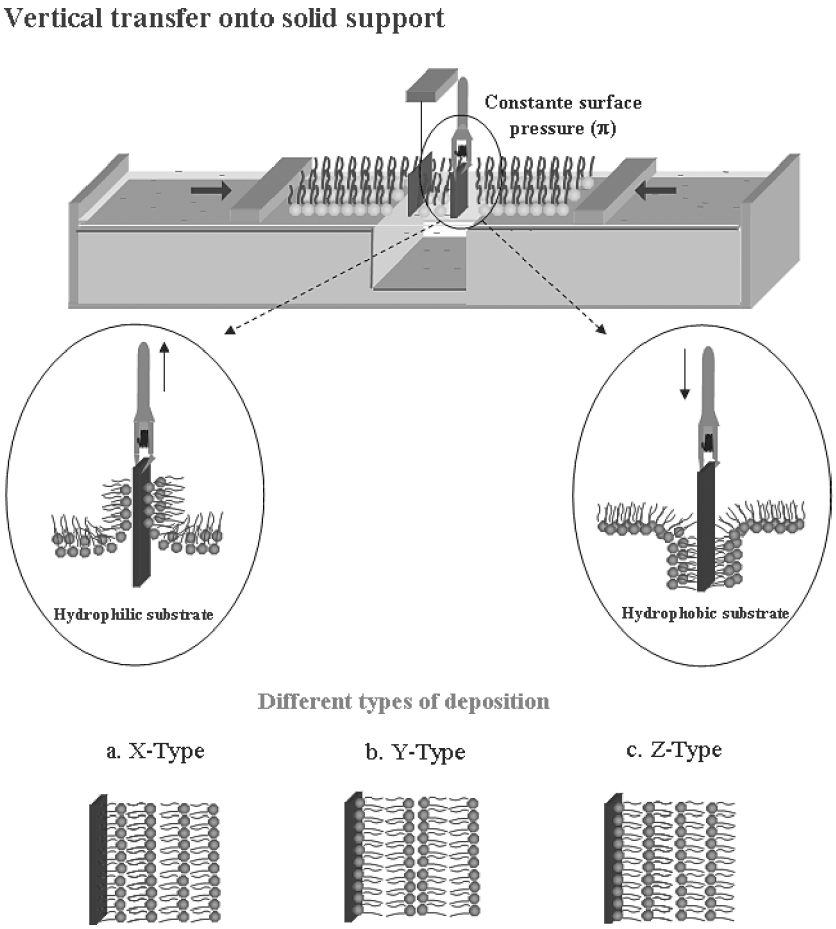

FIGURE 2.7. Langmuir-Blodgett deposition.

type can change as the LB films are built up. XY-type films refers to deposition in which monolayers are completely lay down each time the substrate is being lowered into the subphase, but partially transferred as the substrate moves up through the monolayer/air interface[77,79,85,161]. Despite extensive experimental evidence and a number of theoretical treatments of LB deposition[34], the detailed mechanisms by which the monolayers are transferred has never been completely explained[146]. Many phenomena noted by the original workers, including the different modes of film transfer and the variation at which different materials can be deposited remain not totally understood.

At this stage of the discussion, it must be emphasized that the diagrams shown in Figure 2.7 are simple sketches, which may not always accurately represent the real molecular arrangements on the substrate. For many fatty acid LB films in Y-type form, the long molecular axes can be inclined to the substrate

normal and the tilt angle will depend on the precise deposition conditions[139]; the molecular tilt may also change from layer to layer. In spite of suggested explanations[79,85], the molecular mechanism of the particular X-type transfer process (but also Z-type) is not yet clarified. Some reorganization may occur in the stacking during or shortly after the dipping process[74]. For instance, simple molecules would invert at some stage during the substrate immersion, when the film is inside the subphase water ('detach-turnover-reattach' mechanism), so that the structure finally produced is essentially identical to that of Y-films. For fatty acids, early experiments using X-ray diffraction revealed that the spacing between hydrophilic headgroups was nearly the same, and equal to twice the hydrocarbon chain length, whether they have been deposited as X-type or Y-type films[15,45,84]. It is noteworthy however that in a general manner, the lipids of biological interest normally deposit as Y-type films. The discussion in this chapter will therefore be restricted to the Y-type deposition.

2.3.1.1. Transfer Process Energy

The Y-type deposition is the most usual mode of multilayer formation for amphiphilic molecules in which the headgroup is very hydrophilic and the tailgroup is an alkyl chain. The transfer process energy during the deposition of a monolayer onto a substrate has been studied in detail by measuring the vertical force on the substrate during the dipping procedure[161]. This measurement has been performed by inserting a microforce transducer between the substrate and the dipping arm. This experiment is very similar to the Wilhelmy plate measurement of the surface tension. The vertical net force exerted onto the substrate during the transfer is the same as that measured by the Wilhelmy plate. However, the contact angle is not equal to zero during the transfer process. After subtraction of Archimedes flotation effects, the work of immersion and emersion of the substrate has been determined by integration of the immersion/emersion force cycle recorded during the transfer. Formal analysis shows that deposition during immersion will only occur if the interaction energy between the tailgroups per unit area is higher than the surface tension in the presence of the interfacial film. This implies that there exists a maximum value of the surface tension γ, that is to say a minimal surface pressure π value, beyond or below which deposition will not occur. Indeed, as we will see in the next section, monolayer transfer is often achieved in the condensed state since a minimum surface pressure is a necessary prerequisite to ensure sufficient cohesion in the interfacial film for an efficient transfer. The measurements of the integrated work of emersion indicate that emersion transfer process requires additional energy due to the headgroup dehydration between interlayers. Accordingly, the condition for deposition at emersion requires a high value for head-head interaction energy per unit area, which must be higher than twice the polar group dehydration energy (dehydration of both interfacing layers) in addition to the surface tension γ at the monolayer/air interface. Therefore, the Y-type film formation appears to be greatly dependent on the fact that head-head interactions are usually stronger than tail-tail interactions. For very hydrophilic headgroups, Y-type is the most

stable deposition mode, since the interactions between adjacent monolayers are either hydrophobic-hydrophobic or hydrophilic-hydrophilic. Hydrophilic interactions are, nevertheless, also dependant on pH variations and the presence of multivalent ions in the subphase. Hence, addition of divalent cations into the subphase and/or modification of pH can modify the type of deposition[8,9,79,136], by changing the extent of dehydration and/or the energy interaction between the interlayer headgroups.

2.3.1.2. Contact Angle Values

The other point that must be highlighted and which modifies the nature of the deposition is the dynamic contact angle value between the liquid covered by the monolayer and the substrate coated by transferred layers (*i.e.* the contact angle value of the *meniscus*). As suggested in early reports for a successful deposition [18], the contact angle must be obtuse (>90°) for the downstroke and acute (<90°) for the upstroke. If the contact angle remains obtuse during the upward motion or acute in the downward direction, no layer will adsorb onto the substrate and the multilayers will built up as an X- or Z-film respectively. Such an hypothesis has been more recently verified by precise measurements of the forces[29], the work[161] of emersion and immersion during the transfer process, and by a precise analysis of the dynamic contact angle during deposition of docosanoic acid monolayers from $CdCl_2$ subphases as a function of pH[9]. The transfer efficiency effectively decreases when the contact angle increases form 0 to 90° on substrate emersion. By extrapolation, the authors determined that no transfer occurs if the contact angle equals 90°. However, as will seen in section 2.3.2, an optimal contact angle of 50°– 60° is required for an optimal deposition of a tightly bound monolayer to the substrate surface at the first upstroke (*reactive deposition*). To obtain an efficient transfer at the downstroke, the contact angle must be slightly higher than 90°. The optimal value is located in the range of 95° to 110°.

Numerous papers report contact angle measurement in order to get insight into the deposition process and to elucidate the molecular rearrangement mechanism arising from the XY- and X-type deposition[34,54,129,130]. Some results indicate that the dynamic contact angle on the upstroke is smaller for the first layer directly transferred on a hydrophilic surface than for the subsequent layers. For instance, the difference between the dynamic contact angles of the film-covered subphase on clean mica and during polar deposition on a lead stearate multilayer has been estimated to be approximately 18°[137]. On the other hand, the angle for the downstroke is established with the first layer. This means that one monolayer is sufficient to mask completely the substrate, and it is in agreement with the observation that the nature of the substrate influences the deposition of the first layer. Indeed, the molecular interactions involved in the deposition of the first layer may be quite different than those responsible for the transfer of the subsequent layers, especially for the hydrophilic interactions. Saint-Pierre and Dupeyrat[161] have reported that the values of the work at the first immersion or emersion according to the nature of the substrate are always different from the subsequent values. For the sake of completeness, we can mention that in some

cases, the difference between the immersion and withdrawal angles decrease with increasing the film thickness for the first 18 layers[159], which is coherent with the possible evolution of the deposition type as the LB films are built up. In a same way, Saint-Pierre and Dupeyrat[161] reported that the work of emersion and immersion transfer vary slowly when the number of transferred layers increases, even for Y-types films. Finally, it is important to notice that variations in the contact angle value that create instabilities in the height of meniscus during the substrate motion leads to a non-homogeneous deposition of the monolayer. Such instabilities have been however recently explored in the nanobiotechnology field for patterning lipidic LB film surfaces[68,117,125].

2.3.1.3. Deposition Ratio

The transfer of the monolayer onto a solid substrate is usually characterised by the *deposition* (also named *transfer*) ratio. To be quantitatively transferred, the monolayer must be held at a constant surface pressure during the deposition process. To achieve this, the barrier advance compensates the surface pressure decrease. This allows the measurement of the *deposition ratio*, which is used as an indicator of the quality of deposition. The deposition ratio is defined as the decrease in the area occupied by the monolayer on the surface water divided by the coated area of the solid substrate. The transfer is the most efficient when the transfer ratio equals one. Such a transfer ratio of unity is often taken as a criterion for good deposition, and under most circumstances the orientation of the molecules on the substrate would be expected to be very similar to their orientation on the water surface[76]. An ideal Y-type film is in turn a multilayer system with a constant transfer ratio of one for both up and down directions[85]. However, it must be highlighted that the transfer ratio is sometimes higher (>1) for the first layer, because of the microscopic heterogeneities of the surface of the substrate. There are also cases where the transfer ratio is <1, but remains constant as the dipping proceeds. This consistent deviation from unity would point out the molecular orientation is changing during the deposition process. However, a value outside the range 0.8 to 1.20 suggests poor homogeneity of the transferred film. Variable transfer ratios are almost always a sign of unsatisfactory film deposition.

2.3.1.4. Advantages and Caution

The LB technique offers the possibility to perfectly control each step of the LB film preparation (monolayer formation, substrate preparation, dipping procedure). The main advantage of the membranes obtained by LB deposition lies in the achievement of a molecular arrangement perfectly organized at the water surface, which can be maintained during the transfer onto the solid support when all the parameters are controlled and optimised. Stringent conditions are required for obtaining reproducible results with Langmuir and Langmuir-Blodgett films. Very subtle changes in the experimental conditions may result in dramatic changes in the deposition process. In this connection,

readers can find a detailed description of fundamental experimental requirements (*e.g.*material purity, selection of suitable spreading solvent of high grade purity, accurate weighing of monolayer material, subphase quality made of ultrapure water, precise subphase temperature control, surface cleaning, trough environment, clean and vibration-free environment for carrying out the experiments, monolayer spreading, substrate preparation) in different reference books[145,158]. The Y structure makes LB films ideal candidates for developing biomimetic models of natural membranes.

2.3.2. Elaboration of Organised Lipidic LB Films

As mentioned above, the Langmuir-Blodgett technique is an attractive method for preparing well-organized and structured films as lipid bilayers. However, the development of highly sensitive methods of surface analysis has revealed some defects in lipid LB films, including disclinations[180], in-homogeneous crystalline domains[133], pinholes [13,14,42,107,156], local collapse[36], vacancies[162], transbilayers and lateral heterogeneities[157]. These defects can be harmful for some applications such as molecular electronic devices which have specific functions at the molecular level. Conversely, some applications, like patterned surfaces, are not disadvantaged by generating discontinuous structures in LB films to create nanoscale stripes and channel patterns in LB bilayers[68,117,124,125]. However, the achievement of biomimetic membranes and their applications in nanobiotechnology, particularly in the development of novel nanobiosensors, cannot be performed if lipid LB films are not perfectly structured and homogeneous.

The molecular organization in the LB film depends greatly on the quality of the floating monolayer. Many types of defects found in the LB films can occur before transfer. Such defects are due to the structure and the loss of monolayer integrity during the time elapsed between the spreading and dipping of the monolayer (ageing time)[11,91,192]. Therefore, as mentioned earlier, the first and obvious crucial parameters will be the homogeneity and the stability of the monolayer at the air-water interface. Once the stability of the interfacial monolayer has been fully studied (section 2.2.5) the transfer process can be addressed.

Additionally, the interfacial changes from an air/water to an air/solid interface can impose some strong attractive interactions between the molecules and the hydrophilic (or less often hydrophobic) substrate, which can modify the molecular packing[71,155,172,173,175]. For some materials, a liquid to solid phase transition can occur in the meniscus region at surface pressures lower than that of the LE-LC transition indicated by the π-A isotherm[154]. Distinct phase changes from a LC phase on the water surface to a closer-packed solid crystalline form on the solid substrate have been also reported[112,113,140,141]. In the same way, some fatty acid monolayers transferred in the LE region often condense to form close-packed LC islands on the substrate[166,167,185]. Consequently, a growth of solid domains can occur in the monolayer that is being transferred or immediately after deposition. This change in the molecular arrangement will sometimes generate

defects, which will then grow up in the multilayer structure if the deposition is epitaxial[107]. In the case of fatty acids, electron diffraction experiments have shown each monolayer has the same local orientation of its molecular lattice as that of the underlying layer [143].

The adhesion of the first layer to the underlying substrate is particularly critical and will determine the quality of subsequent layers. This adhesion depends on the nature of the substrate and the attraction forces between the lipid head (or tail) group and the hydrophilic (or hydrophobic) substrate. Pre-treatment of the substrate (*e.g.* silanization, vacuum metal deposition, metal oxidation, lipid pre-coating) can favour the attachment of the first monolayer. Onto metallic substrates, for instances, there may be an ion exchange between ionisable polar group (like those of fatty acid salts) and the thin oxide layer on the substrate surface[144]. Consequently, a strong chemical bond can anchor the polar groups of the first layer to the substrate surface, creating adhesion so strong that only chemically destructive treatments can remove it. It is likely that under such a condition, the chemical and physical structure of the first monolayer will be different to that of subsequent layers. However, for subsequent monolayers transferring onto existing film, the deposition will be homogenous[145].

Mainly two parameters have been identified to be crucial for a high quality deposition: the *deposition speed*, from which depends the quality of the molecular interactions between the substrate and the layers, and the *transfer surface pressure*, from which depends the lateral cohesion of monolayer, but also its homogeneity.

The speed at which the substrate is moved through the monolayer can be different between the up- and down-stroke, and between the first layer and the subsequent ones. As the substrate is lowered into the subphase, it can be moved quite rapidly without affecting the monolayer transfer. In this case, the deposition of the monolayer mainly depends on the hydrophobic interactions and consequently on the transfer surface pressure (section 2.3.1). Conversely, the rate at which a substrate can be withdrawn from the water partly depends on the dynamic properties of the floating monolayer and partly on the rate at which the liquid subphase drains from the monolayer/solid interface[76]. For example, a highly viscous monolayer will be unable to adjust itself so as to maintain a homogenous film in the neighbourhood of a rapid moving substrate. The drainage of the subphase, not only due to gravity, is the result of the adhesion of the monolayer onto the solid material acting along the contact line with the monolayer and driving out the water film (headgroup dehydration). A *reactive deposition* thus occurs when molecules can adsorb spontaneously onto the substrate at the same speed as a new clean area becomes available during the withdrawal of the substrate. In this case, the transfer is accompanied by draining of the subphase, no subphase entrainment occurs and the monolayer becomes tightly bound to the solid, expelling the water layer rapidly. Under this condition, the dynamic contact angle formed by the water meniscus against the solid substrate as it is withdrawn from the subphase (referred as the "zipper angle" by Langmuir in 1938) is around 50°–60°[53] as earlier observed by Langmuir[102].

If the speed at which a new clean substrate area is created during the substrate removal is higher than the rate of the process of this molecular adsorption, the monolayer is practically forced onto the substrate and subphase entrainment occurs (*non-reactive deposition*). Under this condition, the meniscus is distorted and advances faster than the precursor film in the front of the monolayer can adsorb on the substrate. The value of the dynamic contact angle decreases and the substrate comes out wet when it is near to zero[53]. In this case, the monolayer shows a poor adhesion to the substrate and waiting for a complete drying of the water-supported monolayer is necessary to avoid the transferred layer to be re-spread at the subsequent substrate dipping. Then, it appears that the rate at which LB films can be built-up is mainly limited by the rate at which ascending substrate sheds water. In molecular terms, it means that the interactions of the molecules in the monolayer frontier with the substrate determine the success of the deposition at a given rate. The rate at which the water layer is expelled during the transfer process has been referred as the deposition speed[53]. It is important therefore to not raise the substrate faster than the speed at which water drains from the solid. The withdrawal velocity of the substrate must be lower than (or identical to) the adsorption process of the monolayer. The drainage speed depends both on the crystallised state (which increases with the time that the monolayer remains on the subphase water, *i.e.* the ageing time),[142] and on the intrinsic viscoelasticity properties of the floating monolayer[28]. Indeed, the surface viscosity must be below an optimal value for successful deposition. If the viscosity is too high the monolayer will be brittle easily broken on withdrawal (or insertion) of the substrate. Such a rigid monolayer presents a lack of flexibility to ensure the torsion necessary for the meniscus formation during the transfer process. A typical speed of 10μm/s to a few mm/s can be used for the transfer of the first layer onto a hydrophilic substrate. Once the first layer is adhering to the substrate, faster speeds can be applied to deposit the subsequent layers (up to several cm/s).

The optimal value of the surface pressure to produce the best results depends on the nature of the monolayer and is often established empirically[76]. However, the LB deposition is traditionally carried out in the condensed phase since it is generally believed that the transfer efficiency increases when the monolayer is in a close-packed state. In that condition the surface pressure is sufficiently high to ensure a strong lateral cohesion in the monolayer (section 2.2.4), so that the monolayer does not fall apart during the transfer process. Although the optimal surface pressure depends on the nature of the material constituting the film, biological amphiphiles can seldom be successfully transferred at surface pressures lower than 10 mN/m and at surface pressures above 40 mN/m, where collapse and film rigidity (brittle monolayers) often pose problems.

For fatty acids, the condensed state is reached for a surface pressure considerably higher than the equilibrium spreading pressure, ESP (section 2.2.5). So, the transfer of the monolayer is often achieved with an overcompressed monolayer, which does not represent an absolutely stable equilibrium system with respect to the bulk crystal phase. Therefore, it must be keep in mind that even if the

monolayer remains in this *metastable* state for an extended period of time, enabling meaningful experiments to be performed, the monolayer integrity must be preserved during the time elapsed for the transfer process (ageing time). In order to develop a highly organized biomimetic LB structure, a rational approach was first proposed in our group to avoid defect appearance in the condensed monolayer as it gets old (ageing). This approach mainly focuses on the method used to achieve the compression. Indeed, the nucleation crystal growth which occurs when a floating fatty acid monolayer ages has been demonstrated to not only depend on the surface pressure but to be also directly related to the compression procedure[126]. The compression procedure defines the early stages of the monolayer compression which affects the monolayer integrity. It is now well-known that aggregation of the solid condensed domains can start in the gaseous phase[43,188]. Thus, in order to obtain homogeneous LB films, the monolayer must be slowly compressed in order to avoid any surface local overcompressions that would be responsible for the crystal defect appearance through a slow collapse mechanism[126]. The precise compression speed depends on the nature of the lipid molecule and its equilibrium surface pressure. Moreover, to improve the monolayer stability during the time necessary for the transfer process, the transfer surface pressure must be poised at the beginning of the solid phase. This optimizes the compromise between the quantitative transfer of the monolayer, which needs a sufficient lateral cohesion, and the number of crystal defects, which can appear in the ageing floating monolayer.

Finally, a new parameter, referred as the *monolayer deposition rate* (R_D), has been introduced so as to preserve the monolayer integrity during the transfer process. This parameter has been defined as the lipid area deposited onto the substrate within one minute. The parameter R_D takes into account not only the dipping rate but also the coated area of the substrate, and it corresponds to the actual velocity at which the monolayer is removed from the water surface. Consequently, R_D controls the monolayer compression rate during the transfer process, which directly modulates the kinetics of the crystal defect appearance in the floating monolayer[57]. Finally, by adding divalent cations and adjusting the pH value of the subphase accordingly, both the stability and the integrity of the floating fatty acid monolayer can be successfully increased to form LB films of very high quality[106]. By using such an optimal transfer procedure, highly organized behenic acid LB films have been obtained on different hydrophilic or hydrophobic substrates[59].

2.3.3. Phospholipid LB Films

In the context of biomimetic studies, LB films made of phospholipids or glycolipids will be more relevant since they are the essential components of biological membranes. Those molecules are complex lipids conmprising two hydrocarbon chains per molecule (not necessarily of the same length) and a large hydrophilic polar headgroup that is more or less hydrated (Figure 2.3). The primary hydration shell surrounding the phospho- and the glycolipid headgroup

is in rapid exchange with the bulk phase. This shell is constituted of 5 to 20 water molecules for phosphatidylethanolamines (PE) and phosphatidylcholines (PC) respectively[56]. The sugar headgroups of monogalactosyldiglycerides have much higher degrees of hydration than phosphatidylethanolamines[164]. Hence, some difficulties have been encountered to transfer phospholipid and glycolipid monolayers. Since the phospholipid and the glycolipid headgroup may have a stronger affinity for the water subphase than for the hydrophilic substrate[153], the first layer can be transferred at the upstroke by wetting of the substrate and slipping of the monolayer[161]. However, a poor adhesion of the first layer on a hydrophilic support leads the monolayer to peel off the substrate and to respread on the water surface at the second immersion in the subphase. A forced-deposited film will be easy to remove due to the lack of adsorption of the monolayer (lack of polar headgroup dehydration). Consequently, this first deposited phospholipid layer may be then stripped off during the subsequent immersion, as it has been once again recently reported by Hughes *et al.* for the transfer of the dimyristoylphosphatidylcholine (DMPC) monolayer[87]. Under such circumstances, despite the repeated dipping, only one monolayer will be deposited on the slide. To solve this problem, Tamm and McConnell[176] have proposed a combined approach to elaborate phospholipid bilayers on hydrophilic substrate, in which the first layer is transferred by vertical Langmuir-Blodgett (LB) deposition and the second one by horizontal Langmuir-Schaefer (LS) method (Figure 2.8). In this latter method, the substrate horizontally oriented with the face coated by the first layer lying parallel to the air-water interface is slowly lowered until the whole face is in contact with the floating monolayer, allowing tail-to-tail interaction. The substrate is then pushed through the interface for the deposition of the second layer[40,72,83,193].

Many phospholipids including phosphatidylcholines[30,87,89,115,128,133,138,156,171,179], phosphatidylethanolamines[13,14,78,89,163,171,179,207,] and phosphatidic acids[37,38,73,116,118,121,179,183] have been examined for LB deposition. Depending on the nature of the phospholipid and on the experimental transfer conditions (*e.g.* cations, nature of the substrate, surface pressure, transfer speed) different types of phospholipid LB films have been described (Y- or Z-deposition). However, as the interactions between the phospholipid headgroups in the multilayer structure are often weaker than the interactions between the polar groups and the water subphase, the forces implied during the transfer process are not sufficient to pull up the monolayer from the water surface, and only few phospholipids such as phosphatidic acids, can form LB multilayers of more than five layers. To overcome such a difficulty, the mixture of different types of phospholipids (phosphatidylcholines and phosphatidic acids for instance) or of phospholipids with fatty acids can sometimes favour the transfer of phospholipidic monolayers[58,78]. Moreover, it must be emphasised that the transfer ratio value for the deposition of the second layer after transfer of the first one onto a hydrophilic substrate (*i.e.* the outer leaflet of the bilayer) is generally lower than the desired value of unity. A typical deposition ratio value for the transfer (at the downstroke) of a second phospholipid monolayer,

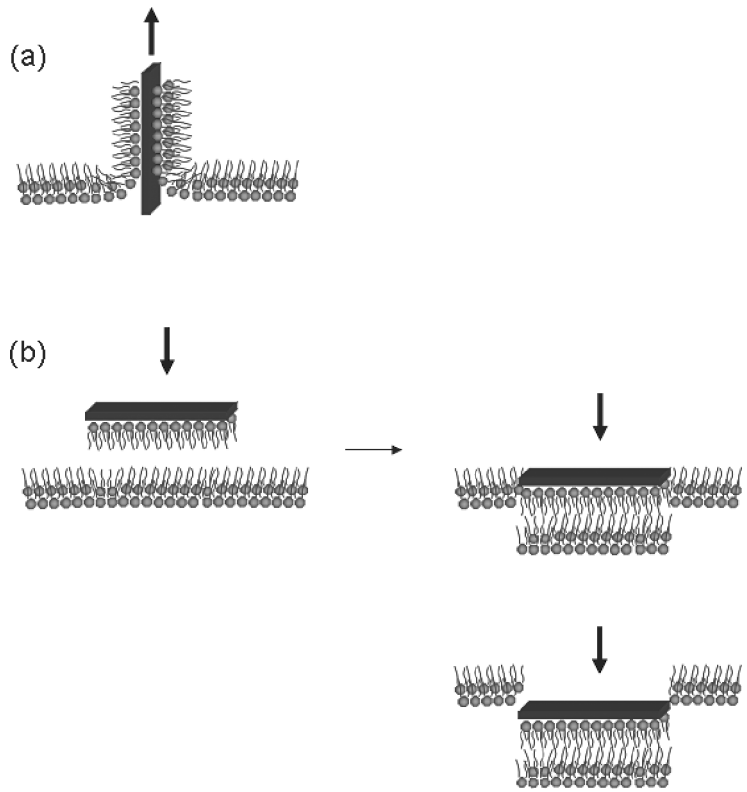

FIGURE 2.8. Mixed monolayer-deposition mode. The first layer is deposited by vertical Langmuir-Blodgett (LB) deposition (a); the second layer is deposited horizontal Langmuir-Schaefer (LS) procedure.

when hydrocarbon chains of the first phospholipidic layer are pointed outside, is often between 0.5 and 1.0[13,30,87,121,125,156]. A possible explanation for this experimental observation has been proposed by Bassereau and Pincet[13]. By using transfer ratio measurements and tapping mode atomic force microscopy (AFM) experiments, these authors suggest that during the deposition of the second monolayer, some lipid molecules of the first monolayer can desorb from the substrate and move over to the interfacial monolayer. This desorption phenomenon has been related to the balance between the adsorption energy of the molecules on the solid substrate and their energy at the air-water interface. In this case, the transfer ratio will reflect the balance between molecules of the interfacial film being transferred onto the substrate and molecules desorbing from the substrate. Therefore, the fact that the transfer ratio value differs from the unit is not a proof (under this condition) for a low transfer quality but it could be equally well interpreted as desorption from the substrate of some phospholipid molecules.

Desorption of lipids of the first monolayer during the transfer of the second monolayer has been shown to be responsible for the origin of the subnanometre bilayer-deep holes in different phospholipid LB bilayers[13,125,156,162] The incoming phospholipids thus cover only the hydrophobic surface of the first monolayer, resulting in lipid-bilayer-covered regions in contact with water that coexist with bare mica. The hole defects range in size from 30 nm (the order size of the AFM tip radius) up to 500 nm[13] and depend on the film transfer pressure, the deposition speed, the number of defects in the lower layer, the type of lipids used and the phase state of the lipids transferred[13,156]. Indeed, it has been reported that the shape of the defects is influenced primarily by the constituents of the first leaflet[156]. The density and the size of the holes decrease as the transfer velocity and the deposition surface pressure increase[13]. Fewer defects appear when the second leaflet is transferred at higher surface pressures[14,128,156]. The percentage of uncovered substrate in bilayers with a second leaflet deposited at a high surface pressure (monolayer in a more condensed phase, typically Liquid Condensed phase) is lower than in bilayers with a second monolayer deposited at a low surface pressure (loosely packed monolayer, typically Liquid Expanded phase). Generally speaking, a higher transfer surface pressure (but see section 2.3.2) leads to the formation of more uniform and tightly packed phospholipid bilayers with fewer pinhole defects[128,199]. Recently, the quantity of the defects (holes of $\leqslant 0.5\,\mu m$) has been correlated to the stability of the bilayers measured by surface force apparatus (SFA)[14]. The condensation state of the floating monolayer that has to be transferred likely determines how many lipids molecules of the first layer leave the substrate during deposition (determining the amount of defects in the bilayers). Since the desorption depends on the balance between the adsorption energy onto the hydrophilic substrate and the affinity for the air/water interface, the decrease of the surface tension when the floating monolayer is compressed limits the affinity of the polar group for the water interface and favours the adsorption onto the hydrophilic substrate[13].

The presence of defects tunnelling both leaflets (holes perforating) in the supported phospholipid bilayers appeared surprising at first because the same lipid systems self-assemble in aqueous solution into tightly sealed vesicular bilayers. But, as reported by Bassereau and Pincet[13], the desorption phenomenon is probably common in any supported bilayer system and has been observed previously by different groups. However, as recently described by Benz *et al.* [14], the formation of holes in supported membranes may be related to the existence of pores in free bilayer membranes[200].

To circumvent this problem of desorption and to improve the transfer characteristics of the phospholipids, it is necessary to enhance the adhesion of the first layer to the substrate. By adapting the procedure optimised for the transfer of a fatty acid monolayer (*i.e.* by adjusting the *monolayer deposition rate* (R_D) according to the size of the immersed substrate area and by positioning the transfer surface pressure at the beginning of the steep rise of the LC phase in the π-A isotherm diagram), dipalmitoylphosphatidic acid (DPPA) and

dipalmitoylphosphatidic acid – dipalmitoylphosphatidylcholine (DPPA-DPPC) mixed monolayers (molar ratio 2:1) have been efficiently transferred in the Y-type form from a pure water subphase onto different hydrophilic substrates[58]. However, depending on the nature of the substrate (fluorine, glass or silicon), an evolution of the deposition type from the Y- (for the two or three first bilayers) to the Z-type has been observed as the number of deposited layers increased. Such an evolution occurs through an intermediate type, now referred as "*YZ-type*". By analogy with the XY-type, "*YZ structure*" is defined to describe the multilayers obtained with a transfer ratio at the withdrawal always close to 1 and a transfer ratio at the downstroke clearly lower than the transfer ratio obtained at the upstroke[58]. This evolution has been directly related to the strength of the interaction between the headgroups of the first layer and the hydrophilic surface. Depending on the type of the substrate, the deposited molecules may adopt different orientations, and as the layers are progressively deposited, the interactions between the tails of the molecules become less efficient, thus decreasing the transfer efficiency during the down-stroke.

Silicon has been demonstrated to be an adequate substrate for the transfer of phospholipid monolayers. In our group, by using the optimal procedure mentioned above, we have transferred up to 21 layers of DPPA and 5 layers of DPPA: DPPC (2:1) in the Y-type form onto a silicon substrate with a transfer ratio of 1.1 ± 0.1 for each of the deposited layers. The role of the substrate mediated condensation (SMC) previously described by Riegler and Spratte for silicon wafer[155,172] had provided a possible explanation for such results. These authors reported that additional adhesive interactions on the substrate can induce some local morphological and constitutional modifications by a variation of the internal pressure, explaining why the first deposited layer was in a more condensed state than the floating monolayer. Therefore, such a SMC effect may enhance the quality of the deposition of the first phospholipid monolayer onto a silicon substrate and the good adhesion of this first layer may orient the molecules in a position favouring the effectiveness of further multilayer stacking. The same denser effect has been recently reported to explain the deposition ratio value slightly higher than the unit for the transfer of phosphatidylcholine (PC) monolayers on silicon surfaces[30]. It must be pointed out that the more hydrophilic the substrate, the better the interaction with the substrate, because a strong hydrophilicity may compensate the strong affinity of the headgroup for the water subphase interface. The difference in the surface hydrophilicity of the different kinds of substrates used for LB deposition can explain the discrepancies between the various behaviours observed during the transfer of phospholipid monolayers. Such behavious include the formation of Y-type bilayers on silicon, and desorption of lipid of the first layer during the deposition of the second monolayer on mica. In fact, the substrate hydrophilicity can favour the adsorption of the molecules on the solid substrate rather than the respreading on the subphase surface and thereby prevent (or not) the partial desorption phenomenon. For this reason, silicon substrates are suitable for obtaining high-quality phospholipid LB films in the Y-form, which constitute an excellent model of the bilayer

structure of biological membranes. In the nanobiotechnology field, elaboration on silicon substrates of phospholipid bilayers as biomimetic lipid membranes appears very attractive to achieve miniaturised bio-electronic hybrids after their functionalisation with proteins or other biological molecules.

The transfer of a phospholipid bilayer system onto a hydrophilic substrate is so far the more relevant approach to elaborate biomimetic membranes. However, it must be emphasised that the transferred bilayers or a part of them (outer layer) can be partially or totally removed from the substrate when the bilayer-covered system crosses vertically a pure air/water interface. This 'detaching' phenomenon is once again due to the balance of the interaction energy between the polar groups on the substrate and their affinity for the air-water interface, and the strength of hydrophobic forces between the hydrocarbon chains. Consequently, a bilayer membrane system deposited on a hydrophilic surface needs to be carefully handled after LB deposition in order to prevent any dewetting problem[13] and to maintain the stability of the outer monolayer[14]. The best way preserve the integrity of the bilayer structure and to avoid any molecular desorption is to keep the supported bilayer under water. After the last transfer at the downstroke, the lipid-covered substrate can be plugged into a small container previously immersed in the subphase, prior to spreading the monolayer[13,14,156,162,163,197,199]. In addition, this produces fully hydrated bilayers, which is the most representative biological situation compare with LB films transferred in dry state (last layer deposited at the upstroke).

2.3.4. Free Supported Phospholipid LB Films

The strong interaction between the first layer and a hydrophilic substrate such as silicon may modify some characteristics of the biomimetic membrane compared with those of the biological one. An example is the fluidity of the supported bilayers. A natural biomembrane is a dynamic, fluid system, where the component molecules have considerable translational freedom and this fluidity is essential for the behaviour of the membrane. The presence of the substrate may constrain the freedom of the component phospholipid molecules within the plane of the membrane, in a manner which may significantly affect the interaction of the membrane system with other biological agents. The supported systems may have structural similarities with a bilayer membrane, but their behaviour may be affected by the tethering effects. Depending on the nature of the biomolecules that will be associated with the bilayer system, these tethering effects may become a major problem. Whereas a single lipid bilayer system is helpful in the studies or in the association of peripheral proteins or proteins residing in the outer leaflet, the global loss of the lipid mobility may be especially harmful in the case of the integration of transmembrane proteins. For this reason, some authors have been interested to deposit a 'double' bilayer of dipalmitoylphosphatidylcholine (DPPC) or distearylphosphatidylcholine (DSPC) onto a silicon wafer (Figure 2.9a). This multi-bilayer configuration has been obtained using the combined Langmuir-Blodgett/Langmuir-Schaefer approach: the three first layers

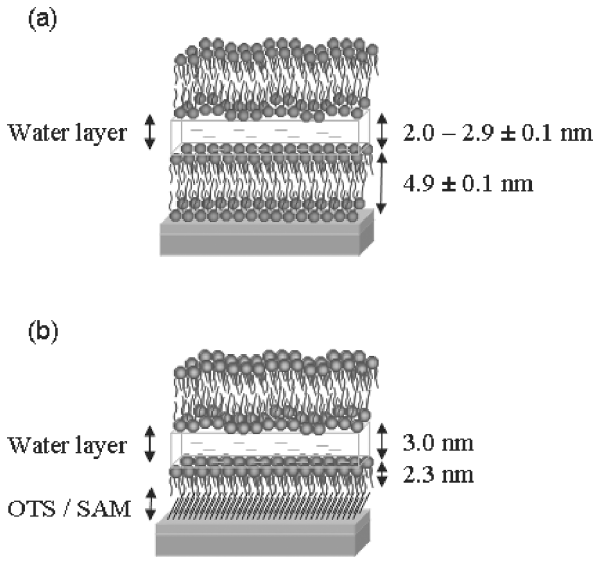

FIGURE 2.9. Schematic diagram of different 'free supported bilayers' (FSB) on silicon wafer. (a) Monolayers 1, 2, 3 were deposited by the Langmuir-Blodgett method; the monolayer 4 was deposited by Langmuir-Schaefer method. The second bilayer, more free to fluctuate, was significantly rougher (0.5–0.6 nm) than the first one[30]. (b) The three outermost phospholipid layers were deposited by Langmuir-Blodgett technique under octadecyltrichlorosilane self-assembled monolayer (OTS/SAM)[88].

have been deposited by vertical LB transfer and the fourth one by horizontal LS deposition, leading the substrate to be immersed in the subphase water at the end of the transfer (section 2.3.3). In this system, the double bilayers retain an intermediate thick hydration layer necessary to limit any substrate tethering affects. The second bilayer, called "*free bilayer*", corresponds to a highly hydrated membrane floating at 2 to 3 nm above the first one[30]. Those authors have obtained some evidence that the thermotropic phase behaviour of the 'free bilayer' mimics that of the vesicle system[48]. More recently,[87] have improved that initial system by using a hybrid self-assembled monolayer (SAM)/double bilayer system, where the lower phospholipid monolayer has been replaced by an octadecyltrichlorosilane (OTS) SAM (Figure 2.9b). As this substrate enhances the transfer ability of dimyristoylphosphatidylcholine (DMPC), it enables formation of a free hydrated bilayer with phospholipid in a fluid state at room temperature. DMPC is a phospholipid which presents a chain melting temperature value (*i.e.* a *phase transition temperature* (T_c) of 24°C lower than those of DPPC (41.5°C) or DSPC (51°C)[22]. Consequently, DMPC forms a Liquid Expanded monolayer at air/water interface. The characterisation of this "*free supported bilayer*" (FSB) by neutron and X-ray reflectivity has clearly demonstrated that the upper bilayer, *i.e.* the '*free bilayer*', is separated from the substrate with a water layer approximately

3 nm thick. Additionally, it presents thermotropic phase behaviours comparable to those observed for DMPC multilamellar vesicles (MLV). Mainly, the authors were able to detect the transition to a ripple phase (P_β'), which is an intermediate phase between the crystalline ordered solid phase (gel phase, L_β') obtained at temperature lower than the melting temperature and the disordered fluid phase (liquid crystalline phase, L_α) in which the chain melts with apparition of *gauche* conformations, obtained at temperature higher than the melting temperature[56]. In the ripple phase, the bilayer adopts a rippled, undulating structure. Therefore, the detection of the transition to the ripple phase is highly significant of the freedom of molecules in that it cannot occur if there is appreciable tethering of the phospholipid molecules to substrate since the ripple phase requires that the phospholipid molecules are able to move normal to the substrate plane[87]. Finally, these authors demonstrate that the structure of this "*free supported bilayer*", in term of area per molecule (A) and bilayer thickness, is identical to that of DMPC vesicles in the gel phase (low temperature). In going from the gel phase to the fluid phase, the bilayer thickness decreases and the area per molecule increases due to the chain melting. The thickness of the intervening water layer decreases across the main transition in the same way as it is seen in multilamellar systems[88].

Hence, it appears that the free supported phospholipid bilayer deposited onto a first bilayer LB film or another mixed bilayer structure (OTS SAM/ LB films) represents a realistic fluid biomimetic membrane. Even if these membranes are not constituted by only one bilayer, they will be able, however, to find applications in the nanobiotechnology field for molecular systems based on membrane fluidity. These applications could include systems incorporating integral proteins like pores or ion channels, design of drug delivery vehicles requiring membrane fusion with the target cell, or molecular recognition of ligands by cell-membrane receptors.

2.3.5. Asymmetric Phospholipid LB Bilayers

Another interesting aspect of the LB film deposition is the possibility to elaborate asymmetric phospholipid bilayers, termed *alternate-layer LB films*. Indeed, in animal and bacterial cells the lipid composition differs from the inner to the outer faces of the plasma membranes. For instance, the inner lipid layer of the human erythrocyte membrane contains most of the phosphatidylethanolamine and phosphatidylserine, whereas the outer leaflet contains most of phosphatidylcholine and sphingolipide[105]. Beside the specific variation which can be encountered in the total lipid composition, asymmetric lipid distribution is a common aspect of the different types of biological membranes.

LB deposition allows building up films containing more than one type of monomolecular layer. In the simplest case, alternate-layer films may be produced by raising the substrate through a monolayer of one material (consisting of molecules of compound A) and then, lowering the substrate through a monolayer of a second substance (compound B). A multilayer structure consisting of

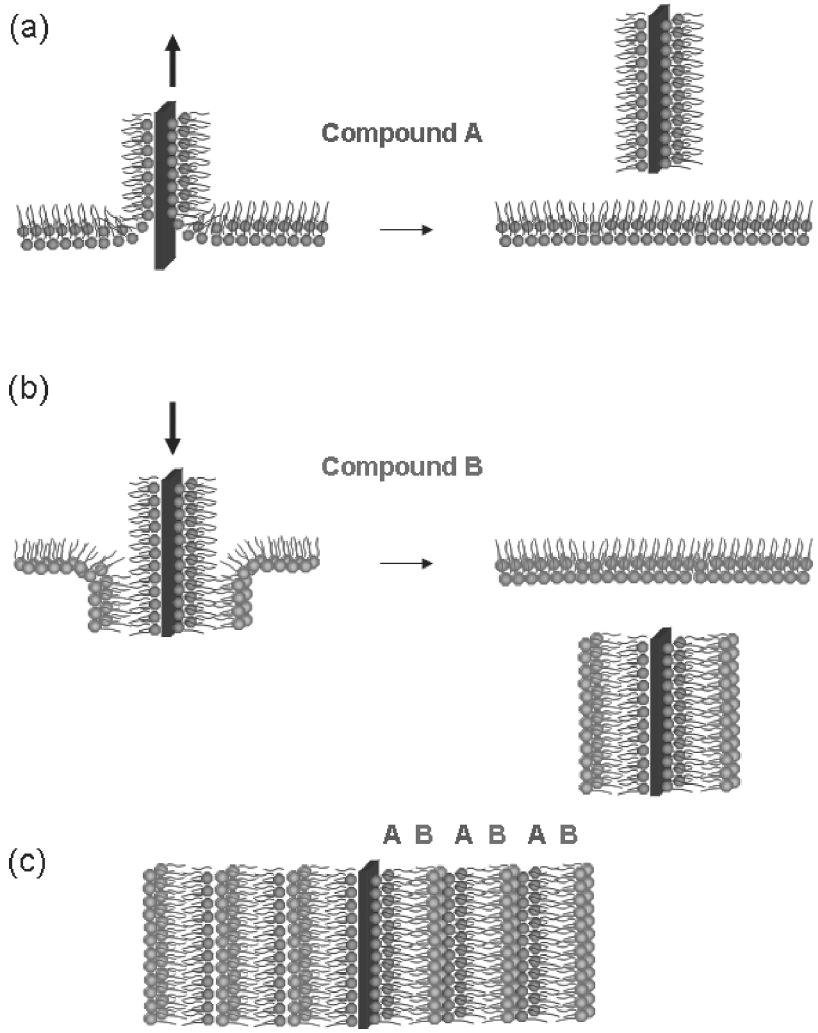

FIGURE 2.10. Alternate-layer Langmuir-Blodgett films. The alternate multilayers were obtained by (a) raising the substrate through the monolayer of compound A; (b) lowering the substrate through the monolayer of compound B, etc… (c) Structure of ABABAB alternate LB multilayers.

ABABAB… layers will be produced (Figure 2.10). In particular, *alternate-bilayer* structures are of great interest in the study of the membrane asymmetry and may find applications in the development of biomimetic bilayer structure. In this case, one monolayer is transferred in emersion (A) and the other in the immersion mode (B).

Production of asymmetric membranes (*i.e.* hybrid supported bilayers) by LB deposition can be helpful for the analysis of lipid phase-separation

and lipid domain formation in mixed bilayers[162,197]. Despite the traditional view of a lipid bilayer as a dynamic, fluid environment, there is increasing evidence that organization of lipids and other membrane components into microdomains, named rafts, plays a crucial role in many cellular functions[2,169,170]. In another respect, asymmetric bilayers can be produced with the first leaflet formed by zwitterionic phospholipids and the second one by anionic phospholipids. Such bilayer structures may have potential applications for protein incorporation, since many of them associate with membranes *via* interactions with anionic phospholipids[156]. A number of recent publications deal with the formation and the characterization of phospholipidic asymmetric LB bilayers of different phospholipid combinations, including DMPE/DOPC[13], PC/PG or PC/PE[156], DSPE/DOPE, DSPE/DOPE-DSPE-MGDG mixture,[162,163], DPPE/DPPC, DPPE/DPPC-GM1 mixture, DPPC/DPPC-Cholesterol-GM1 mixture[197], DPPE/SPM-DOPC, DPPE/SPM-DOPC-Cholesterol mixture, DPPE/SPM-DOPC-Cholesterol-GM1 mixture[199], DPPE/ DLPE[14]. These different types of combinations has been mainly characterized in terms of structural homogeneity and lipid phase-segregation. An interesting point is that the asymmetric bilayer is more homogeneous (with fewer defects) when the first layer is composed of phosphatidylethanolamine (PE) compared with phosphatidylcholine (PC)[13,156,197]. As mentioned above (section 2.3.3), this result is partially due to the lower number of defects in the original PE monolayer and to a resulting lower probability of losing some molecules of the bottom monolayer on second transfer[197]. Specific properties of PC cause polygonal patterns in supported bilayers[156]. PC is known to have a bulky headgroup with a larger cross-sectional area than that occupied by two saturated acyl chains. In hydrated bilayers (in condensed phase) this causes packing constraints, resulting in tilted acyl chains[80]. Hence, supported PC monolayers consist of multiple ordered domains with lipids in a tilted conformation in different directions from one domain to another, thus creating disordered line defects on the borderlines between the different domains. From these weak line defects, PC molecules preferentially desorb upon passing through the second monolayer, generating more defects in the bilayers[156]. This does not occur with PE which has a smaller headgroup and for which the acyl chain is straightforward.

The success of the biomimetic membrane applications in nanobiotechnology relies on the understanding of the fundamental properties of the membrane itself. The production of phospholipidic asymmetric membranes will be an innovative way to develop a new kind of biomimetic membranes that may open new opportunities to integrate recognition biological systems. Finally, it can be mentioned that asymmetric membrane composed of biological molecules can additionally possess some specific properties due to the non-centrosymmetric (*i.e.* no plane of symmetry)[182]. A pyroelectric effect has been reported by developing alternate-layers LB films of different phospholipids (DPPA, DPPS, DPPC, DPPE), long-chain fatty amine (docosylamine) and fatty acid (22-tricosenoic acid)[147].

2.4. Functionalisation of Lipidic LB Films: Specific Features

The functionalisation of the structural biomimetic membranes by association or incorporation of macromolecules presenting specific biological activities such as enzymes, antibodies or specific ligands is one of crucial steps for multiple applications in nanobiotechnology. This section will be concerned with a brief overview of methods enabling functionalisation of LB membranes, based on specific features of the Langmuir-Blodgett technology.

Due to the fact they are transferable on various types of substrate, LB films present great advantages for the development of novel nanobiosensors. Like the other systems mimicking the biological membrane, their structural organization (highly ordered molecular array) and their ultrathin thickness (few nanometers) are the main criteria for the design of ultra-rapid micronic sensors operating at the molecular level and of further developments of "smart" sensors and biochips. However, the interest of LB films is not limited to these structural aspects. Specific advantages include (i) the ability to achieve in a one-step procedure the elaboration of a bioactive sensing layer and its association with the transducer, *(ii)* the low enzyme amount needed for the membrane preparation, *(iii)* the possibility to work at ambient pressure and temperature avoiding thermic treatments arising in the micronic system design (which can denature the biological compounds), and *(iv)* the ability to modulate the sensor performances (detection limit, sensitivity, dynamic range) in varying the number of the deposited proteo-lipidic layers[6,31,108,127,135,194].

The successful incorporation of biological compounds retaining their activity in LB films remains the crucial step as well. Several approaches to build up supported lipid LB bi(multi)layers containing proteins have been reported. Principles, advantages and inconveniences of such specific methods based on LB technology are briefly listed in the following sections.

2.4.1. Protein Association with the Floating Monolayer before LB Deposition

One of the most commonly used approaches derives from the procedure developed to study protein-lipid interaction with a Langmuir monolayer[25,39,49,184,206]. It corresponds to the adsorption of the protein from the subphase onto the interfacial film before transfer of the mixed proteo-lipidic monolayer (Figure 2.11). For 25 years, several bioactive protein-lipid LB films have been produced by using this approach[12,33,46,50,109,123,148,152,160,176,191,205,206] and some of them have been studied with regard to their potential applications in biosensing devices[6,7,31,47,127,174,194,201,202,204]. However, additionally to the relatively large amount of proteins required in the Langmuir trough, some drawbacks must be pointed out[81]. If the monolayer is prepared on a subphase containing the dissolved protein, a layer of denatured protein may

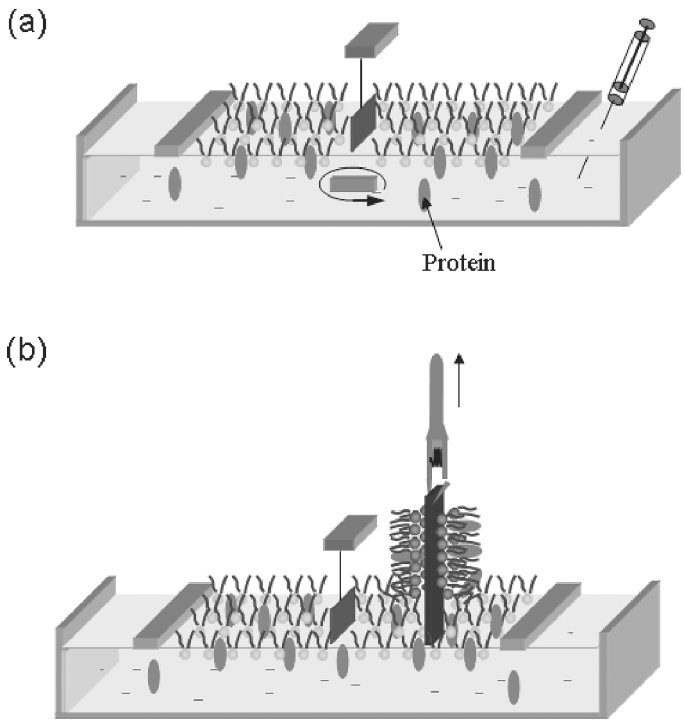

FIGURE 2.11. Protein insertion in the floating monolayer before Langmuir-Blodgett deposition.

form on the water surface and may mix with the lipid layer. If the protein is injected into the subphase after lipid monolayer formation, the protein may penetrate the monolayer close to the injection point and hence, may not distribute homogeneously in the interfacial film. In this case, multipoint injections or injecting the protein progressively under the monolayer may circumvent this disadvantage.

In order to avoid protein denaturation at the air-water interface, the possibility to directly spread the enzyme or a protein/detergent mixture on the surface of a floating lipidic monolayer has been reported[81,108,135]. Using these techniques, homogeneous proteo-lipidic monolayers have been formed. The formation of a protein/synthetic dialkyl amphiphile complex, soluble in an organic solvent (benzene or chloroform) and directly spreadable at the air-water interface, has been also used to prepare active and stable proteo-lipidic monolayers that preserve the enzyme activity and are transferable onto an electrochemical transducer[131,132].

Finally, the presence of the enzyme in the mixed monolayer could modify its transferability properties[31,191,206]. The surface pressure needed for the transfer procedure is not always adapted for the enzyme association. The expulsion of the

protein is often encountered at high surface pressures. Furthermore, the presence of the protein can induce a poor monolayer adhesion on the substrate, which can lead to a peeling-off at the subsequent immersion.

2.4.2. Protein Association onto Preformed-Lipidic LB Films

Another approach consists in the adsorption of the enzyme onto pre-formed LB films[3,4,32,61,62,104,119,128]. The main advantage of this procedure lies in the possible interaction of the enzyme with a hydrophobic or hydrophilic surface depending on the number of the deposited layers, thus allowing the control of the enzyme environment. Likewise, this approach allows the control of the thickness and the homogeneity of the LB films harbouring the enzymes. Nevertheless, the release of protein molecules due to the weakness of their association with the lipidic surface remains a major drawback[120] and is often the main reason which explains the poor reproducibility of responses of LB membrane-based sensors[5]. In order to avoid desorption, some authors have proposed to covalently immobilise the enzyme on LB film surfaces by the use of crosslinking agents[178,181]. The stabilisation of the proteo-lipidic LB films by reticulation after transfer with glutaraldehyde vapour has been also investigated[191,204]. However, covalent attachment to the lipid structure may induce changes in the protein conformation, which can lead sometimes to a loss of its biological activity.

Another alternative to limit protein desorption and avoid covalent immobilisation is to cover the protein molecules by transfer of an outermost layer after enzyme adsorption. This procedure referred as "*inclusion process*" allows the sandwiching of the enzyme in a hydrophobic or a hydrophilic environment while keeping the homogeneity of the supporting layers[60]. Alternate-layer LB films such as monolayer 1/adsorded protein layer/lipid monolayer 2, have been also considered[16]. The specific features of these latter methods are the possibility to easily vary the types of monolayers, the conditions of their deposition and of protein adsorption during the film preparation process. The control of these parameters allows improving the stability of the mixed LB films to preserve the protein activity[16].

Finally, the appropriate choice of the monolayer onto which protein should be adsorbed can advantageously limit the protein detachment. The use of asymmetric LB bilayers of zwitterionic and anionic phospholipids may present an advantage for an efficient protein association (section 2.3.5). Moreover, an appropriate monolayer may prevent the protein denaturation, which can occur by adsorption onto high-surface tension hydrophilic surfaces.

Whether the protein is associated to the LB membrane before or after the monolayer deposition, it stays randomly adsorbed on the surface of the lipid layer. The control of the protein incorporation in a defined orientation similar to the biological membrane, where the protein association in/on the lipidic leaflets determines their own orientation for an optimal functionality, remains a

crucial challenge to achieve functionalized biomimetic membranes. The building-up of proteo-lipidic LB bilayers possessing properly oriented recognition sites constitutes a promising model for further developments in biomimetic sensing applications.

2.4.3. Oriented Protein Association in Lipidic LB Films

In order to overcome the problem of protein orientation associated with biomimetic membranes in general, and with lipidic LB films in particular, several attempts have been recently performed. These include the covalent coupling of antibody fragments through disulphide bridges to a lipid linker embedded in phospholipid monolayers[90,186,187], or the immobilisation of histidine-containing proteins onto metal ion chelating lipid monolayers[96]. However, in this latter case, several defined binding orientations could be obtained depending on the spatial distribution of histidine units on the surface of the protein. With the aim of designing functionalised biomimetic membranes with an unique recognition site orientation, another approach has been recently proposed, which involves the insertion of a non-inhibitory monoclonal antibody (IgG) in LB films as an anchor. This anchor is able both to sequester a hydrophilic protein (soluble enzyme) in an oriented position at the surface of the lipidic matrix (avoiding adsorbed protein denaturation) and to preserve biological (enzyme) activity over few months (Figure 2.12)[69,70].

In order to ensure the functional orientation of the antibody in the glycolipidic LB membrane, our group has developed an original strategy which is based on an adapted combination of liposome fusion at an air-buffer interface and Langmuir-Blodgett technology. This strategy exploits the possibility to spread proteo-lipidic liposomes at an air-buffer interface, which are able to disintegrate and to form a mixed proteo-lipidic monolayer[64]. After compression, the mixed monolayer can be transferred by LB deposition[65]. The main interest of forming proteo-lipidic vesicles prior the interfacial film formation is to favour the creation of optimal proteo-lipidic interactions in order to improve the protein retention both in the interfacial film and in the LB films, without implying covalent bonds. The governing idea of this method is to include the antibody (soluble protein) in pre-formed lipidic vesicle membranes able to carry it towards the air/buffer interface directly in a lipidic environment[63].

The building-up of the functionalised LB biomimetic membrane depicted in figure 2.12 has been explained by the strong interactions occurring between the fluid lipid matrix, which corresponds to a synthesised glycolipid having high hydrocarbon chain fluidity, and the immunoglobulin, which is a glycoprotein. On the one hand, weak (but favourable) carbohydrate/carbohydrate hydrophilic interactions could exist between the glycolipid headgroup and the glycan moiety of the IgG molecule (located in the hinge region). On the other hand, hydrophobic interactions could embed the hydrophobic Fc fragment of IgG in the lipid moiety of the glycolipid leaflets; this embedment is favoured by the high fluidity of the glycolipid hydrocarbon chains allowing their conformational adaptation[70].

These specific interactions initially formed in the vesicle membrane are preserved during the interfacial vesicle disintegration and lead in turn to a preferential orientation of the IgG molecules in the supported bilayer structure[70]. After immunoassociation, the enzyme will be retained at the surface of the bilayer structure in a well-defined orientation[69].

This functionalised biomimetic membrane has been shown to be structurally stable and able to retain enzyme activity for a long period of time (over a period of 82 days)[69]. To our knowledge, such a high stability has never been reported previously for an immobilised enzyme onto Langmuir-Blodgett films. Furthermore, the typical enzymatic behaviour of the enzyme retained at the surface of the biomimetic membrane has clearly demonstrated the potential

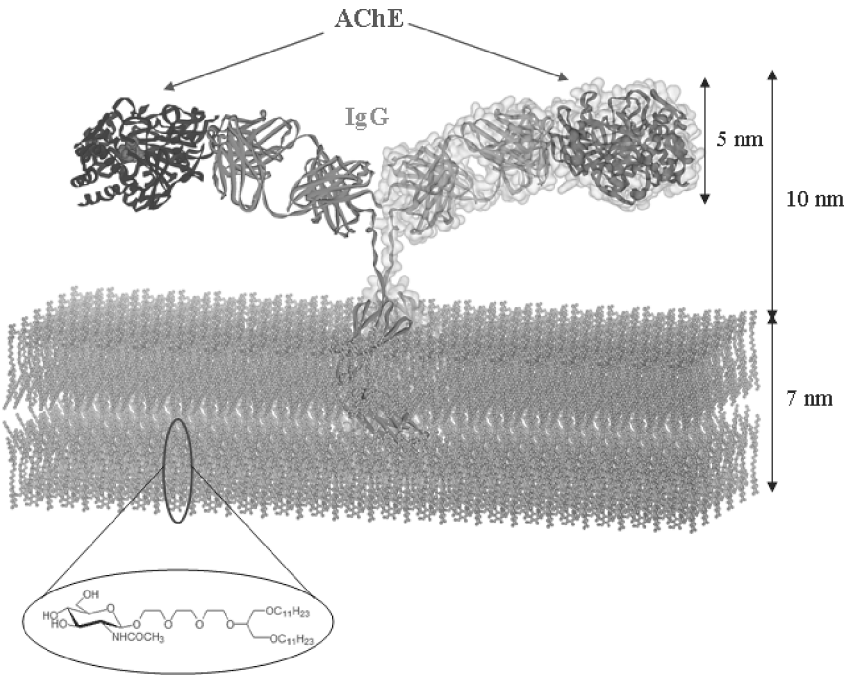

FIGURE 2.12. Functionalized bilayer Langmuir-Blodgett structure with unique recognition site orientation. This biomimetic membrane has been obtained by using an adapted combination of liposome fusion at an air/buffer interface and the Langmuir-Blodgett technology (see text). The lipidic bilayer is constituted of a neoglycolipid presenting highly-fluid hydrocarbon chains. The antibody is embedded in the bilayer structure by the way of (i) favourable carbohydrate/carbohydrate interactions between the glycan moieties of the antibody (IgG) molecule and the glycolipid head group and (ii) probable hydrophobic interactions between the Fc fragment of the immunoglobulin and the lipid moiety of the glycolipid leaflets. The acetylcholinesterase (AChE) is associated to the functionalized bilayer structure, after transfer, by immuno-association. This functionalized biomimetic LB membrane has been shown to be stable more than 3 three months[70].

usefulness of such a functional molecular assembly for biocatalysis investigations in a biomimetic environment[70].

Interfacial spreading of proteo-vesicle together with Langmuir-Blodgett technique appears thus to be an efficient method for developing functionalized biomimetic LB membranes with favourable orientation of recognition sites. The combination of these methods, based on the self-molecular assembly of biomolecules, allows both the insertion of the protein presenting specific recognition properties with a pre-determined orientation in the vesicle membranes and the enhancement of the proteo-lipidic organization by using lateral compression.

2.5. Trends and Prospects

For several years, self-assembly properties of biomolecules received more and more attention because of their ability to spontaneously organize into nanostructures, which allows mimicking the living cell membranes. As attested by the number of recent papers quoted in this chapter, LB technology is a powerful method to elaborate functionalised biomimetic membranes. Different aspects of the biological membrane, like fluidity or asymmetry can be preserved, but the most promising outcome resides in the possibility to orient functional macromolecules in the bilayer structure. By the way to be directly prepared at the surface of different kinds of solid materials, LB membranes present some real advantages for applications in nanobiotechnology and applied nanobiosciences. A direct association with active surfaces constitutes an attractive opportunity for designing novel nanosensors. The intimate contact between LB membranes and effective transducers, allowing recognition and signal transduction events in a single device is without doubt, a very promising way for the development of biomimetic nanosensors and minute investigations of biological processes at the molecular level.

Finally, the possibility to incorporate amphipathic biomolecules in LB membranes offers the opportunity to diversify the number of new organized nanosensing bilayer which can be devised. Actually, a precise knowledge of the hydrophilic and hydrophobic domains of macromolecules will allow different kinds of molecular assemblies to be designed for fundamental and applied investigations.

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