Anales Acad. Nac. de Cs. Ex., Fís. y Nat., tomo 64 (2012): 30-53.

THE STRUCTURAL DYNAMICS OF BIOMEMBRANES

Bruno Maggio

Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC- CONICET).
Departamento de Química Biológica, Facultad de Ciencias Químicas,
Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende,
Ciudad Universitaria, X5000HUA Córdoba, Argentina
E-mail: bmaggio@fcq.unc.edu.ar

Abstract

Biological membranes are constituted by a large diversity of self-assembled lipid and protein molecules. These structures show a varied polymorphism and represent one of the more complex and less understood states of matter whose properties underlay and determine biochemical reactions in biomembranes and, as a consequence, all cellular functions. The chemical moieties and interactions at the local level affect the molecular shape that constrains the overall structure and topology of the surface, the membrane-associated enzymatic reactions and the possibilities for biomembrane fission and fusion in cell division and endo/exocytosis. When the molecular structure and interactions are taken into account it becomes inevitable the emergence of curvature tensions and thermodynamic compensations in the self-assembled system. In turn, the lateral and transverse tensions developed at the supramolecular level restrict and modulate the local interactions and molecular organization in a multidirectional communication. In order to describe and understand the structural dynamics of membranes a combination of approaches involving differential geometry, topology, electrostatics, rheology, and statistical thermodynamics is required, constructed over results provided by the molecular biophysics of lipids and proteins in polymorphic meso-structrures.
Mixtures of glycerophospholipids, sphingolipids and sterols can transduce molecular-topologic information with the capacity to influence simultaneously the biomembrane behavior in three synergic hierarchical levels: a) Local molecular properties, molecular shape and near-neighbor self-organization; b) Tensions over the lateral and transverse planes, phase state, miscibility and topology; c) Longrange supramolecular dynamics determining ligand/membrane recognition, surface biocatalysis and membrane recombination.

Key words: lipid monolayers; lipid bilayers; sphingolipids; phospholipases; sphingomyelinase;
membrane structure.

Resumen

La dinámica estructural de biomembranas. Las membranas celulares contienen una gran diversidad de lípidos y proteínas. Estas estructuras presentan un polimorfismo dinámico constituyendo uno de los estados más complejos de la materia que subyace y determina reacciones bioquímicas y, por consiguiente, todas las funciones celulares. Las estructuras químicas e interacciones a nivel local influencian la forma molecular y determinan la estructura, la topografía, las reacciones enzimáticas y la posibilidad de recombinación (fisión, fusión) de biomembranas. Cuando se toman en cuenta la estructura y forma de las moleculas

autoorganizadas es inevitable la emergencia de tensiones de curvatura, viscoelásticas y las restricciones termodinámicas que ello impone al sistema autoorganizado. A su vez, las tensiones desarrolladas en la estructura supramolecular condicionan y regulan las interacciones y la organización molecular locales, en un complejo lenguaje multidireccional. Para describir y entender las estructuras y la dinámica de membranas biológicas se requieren elementos de geometría diferencial, topología, reología y termodinámica estadística combinados, construidos sobre la base de resultados aportados por la biofísica molecular de lípidos y proteínas en mesoestructuras polimorficas. Mezclas de glicerofosfolípidos, esfingolípidos y esteroles tienen capacidad de transmitir y traducir información molecular-topológica con capacidad de influenciar simultáneamente el comportamiento de biomembranas en tres niveles jerárquicos
sinérgicos: a) Propiedades moleculares locales, forma molecular y auto-organización de corto alcance; b) Tensiones sobre el plano lateral y transversal, estado de fase, miscibilidad y topología; c) Dinámica supramolecular a largo alcance determinante del reconocimiento de ligandos y otras membranas, biocatálisis de superficie y recombinación de membranas.

Palabras clave: monocapas lipídicas; bicapas lipídicas; esfingolípidos; fosfolipasas; esfingomielinasa; estructura de membrana.

Introduction

Different to the rather static picture still conveyed by most models shown in textbooks, membrane structures have revealed an extraordinary polymorphism with complicated dynamics. Understanding their behavior on molecular terms actually means dealing with physico-chemical properties of interfaces, and vectorial self-structuring of amphipathic molecules. This is because the membrane behavior corresponds to that of anisotropic viscoelastic fluids, whose structural dynamics involves many correlated parameters.

These determine and control the surface miscibility of components, phase state, domain segregation, surface curvature and structural topology, as well as exchange of molecular information along and across the membrane. Understanding the molecular principles underlying the self-structuring of these polymorphic liquid–crystalline phases, one of the most complex and least understood states of matter, adds further complexity and limitations because molecular control at each level of inspection is required in order to reach valid conclusions.

It might be interesting to remark that, as summarized elsewhere [1], the study on molecular terms of self-organized amphipathic molecules, such as lipids in aqueous media is a very old story. The phenomenon was empirically known since milleniums ago from the surfaceactive properties of oils and fats as reported on cuneiform tablets in the Hammurabi Code (about 1700s BC). Greeks and Romans began using it about 1,000 years later and organic monolayer films were known in the far-orient according to recipes for the old Japanese printing art *suminagashi*.

Scientifically-based studies of lipid organization using artificial model membranes span more than two centuries. According to the Philosophical Transaction of the Royal Society, in 1774 Benjamin Franklin prepared the first monomolecular layer of oleic acid at constant area on the surface of a pond in Clapham Common, a neighborhood near London. Till this day, most automated monolayer equipments are based on an ingenious method developed by Mrs. Agnes

Pockels in 1881 and Lord Rayleigh published a paper in 1890 suggesting that olive oil films were "of molecular thickness", between 1 and 2 nm thick.

A milestone for biology was established with the monolayer experiment of Gorter and Grendel [2] in 1925 from which the very idea of a lipid bilayer as the fundamental structure of cell membranes was derived using only the transverse half of a membrane. Thus, it should be recognized that interest and experiments in the now fashionable "Molecular/Structural Biology" were actually initiated with studies of lipids and artificial model membranes at the air–water interface and not with cellular systems as most publication media misinformation conveys to the unaware.

Using such biomimetic systems, increasing experimental evidences began to point out during the late 1970s the extraordinary synergy of molecular factors involved in membrane behavior (for references see [3-5]). In 1981 [6] our laboratory initially reviewed this point with an emphasis on glycosphingolipids and their interactions with membrane components of nerve cells furthering this concept with integrated evidences in a number of reviews since 1994 [7-10].

One of the essential features of the major classes of biomolecules, such as lipids, proteins, nucleic acids, proteoglucans and polysaccharides, is their capacity to self-assemble, thus spontaneously acquiring short- and long-range organization in a vectorial and collective manner.

Practically all we know on the structure and dynamics of cell membranes derives from a rich background of research on few biophysical properties of membrane molecules. The whole membrane becomes involved because it is thermodynamically unavoidable that molecular properties become amplified, balanced, translated, and modulated by and to the supramolecular level of the membrane structure. Variations of local molecular interactions will determine lateral miscibility, phase state, and surface topography; besides, molecular geometry, electrostatics and viscoelastic deformations control surface curvature and topology of the lateral and transverse planes of the membrane. This configures spontaneously organized bio-electro-mechano-chemical molecular nano-micro-processors inherently endowed with highly specific and spatially oriented molecular recognition and reactivity, capable of storing and transducing molecular information among all those levels. Through these properties, events at the atomic (chemical groups) are amplified and modulated up and down amongst the molecular-supramolecular-topographic-topologic levels acting as dynamically-regulated metabolic and structural manifolds.

One of the major parameters that influences, and in turn responds to, variations of molecular packing and surface structuring is the two-dimensional (lateral) surface pressure at the interface. The average surface pressure results from a balance of repulsive electrostatic or hydration forces along the hydrophilic surface, and attractive interactions (mostly dispersion London van der Waals type) or steric/conformational factors in the hydrophobic region of the membrane. The amplitude and frequency of lateral pressure fluctuations depend on the molecular thermal energy, the in-plane elasticity and the surface viscosity and are usually coupled to oscillations of membrane curvature (Fig. 1). There is thermodynamic correspondence between bilayer and monolayer interfacial properties over all the range of possible surface pressures, and average values of 30-35 mN/m have been correlated to those in natural membranes and lipid bilayers [11, 12]. However, this parameter exhibits large fluctuations that can span more than 15 mN/m [13] occurring over time intervals of micro- to milliseconds.

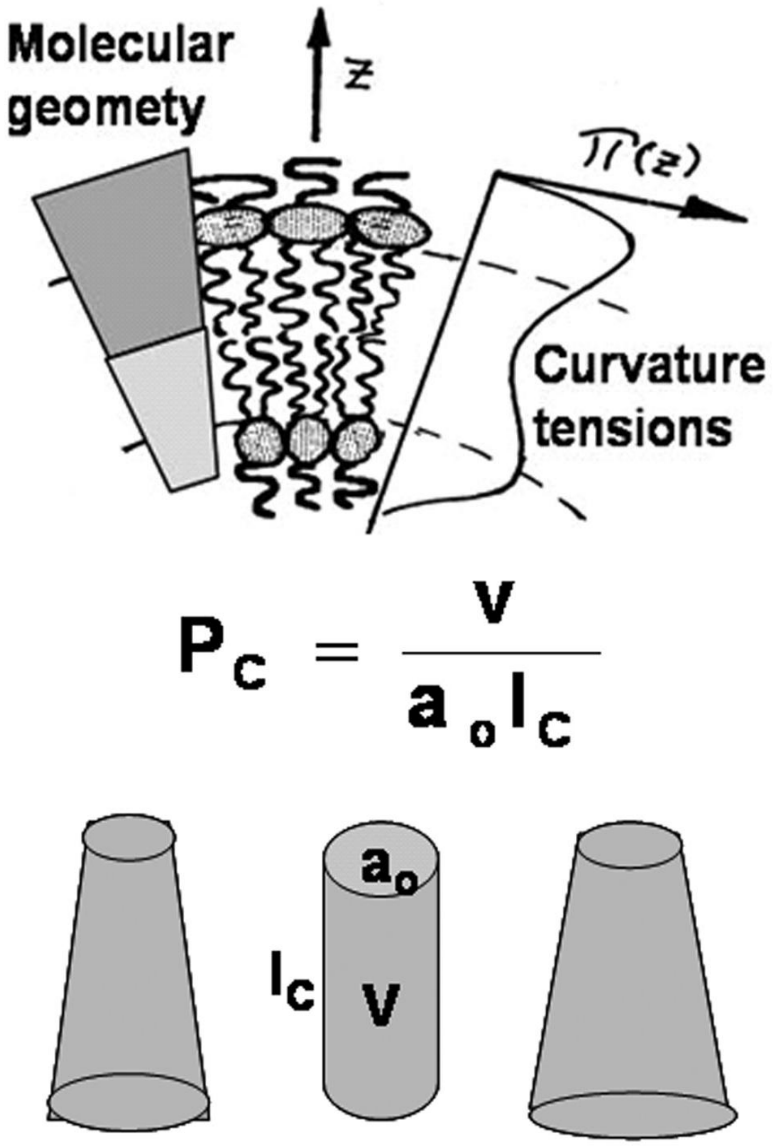

Fig. 1. Upper panel : cartoon showing the curvature tension effects induced by dissimilar molecular geometries of amphipathic molecules. Lower panel: the molecular geometry of amphipaths can be specified by controlled variations of the relative magnitudes of cross-sectional molecular area (ao), and the length (lc) and volume (v) of the hydrocarbon moiety.

The fluctuations of lateral pressure are inherently included in the average phase state of the interface, while the in-plane elasticity and viscosity can absorb, dampen or amplify the local variations. If the compressibility of the surface was approximately homogeneous along the lateral plane, the stress generated may propagate radially as uniform dampened oscillations. However, if less deformable barriers (such as segregated phase-condensed domains) were encountered, the lateral tensions may be relaxed to isothermal phase transition, coupled or not to variations of local interfacial curvature. The kinetics for formation/relaxation of relatively small phasesegregated domains is within the time range of surface pressure fluctuations [14] and this can be orders of magnitude faster than the catalytic rate of membrane-associated enzymes [15].

Factors that affect intermolecular packing can be simultaneously transduced to curvature alterations of the interface and vice-versa. The polar head group size, hydration and sectional area exposed to the aqueous phase, in relation to the volume and length of the

hydrocarbon moiety, can determine molecular shapes different to a cylinder and more similar to a cone [16]. Thus, domains enriched in complex GSLs bearing a bulky and negatively charged oligosaccharide tend to increase positive curvature (bending away from the aqueous interface). These effects occur spontaneously whenever lateral pressure fluctuations may drive the molecular packing areas to exceed the critical limit that is compatible with the interfacial curvature..

Another manner of transmitting the molecular information to higher structural levels is by whole topological rearrangement involving non-bilayer phases. In systems constituted by more than one type of molecules, there may be compensations enhancing or alleviating stress that are reflected in the overall topology (Fig. 2). Small amounts of HI(micellar)-phase-forming lipids in binary or ternary mixtures with other lipids that spontaneously tend to form nonbilayer HII (inverted micelle or bicontinuousn cubic)-phases cause facilitation, impairment, or elimination of the HII-phase structure [17-19]. A sphingolipid such as Cer, some fatty acids and lyso-derivatives or glycerides have a small polar head group in relation to the hydrocarbon chain volume. Such feature favors self-organization into structures with negative interfacial curvature such as HII-type of phases which facilitate lipid flip-flop across the bilayer. Besides, HII-phase like regions act as key structural intermediates for inducing reduction of membrane symmetry [20,21] leading to cell and lipid bilayer membrane fusion or fission (Fig. 2). The latter process is an important element for controlling release of bioactive compounds from self-assembled liposomes [22]. Several sphingolipids can induce hemi-fusion, whole bilayer vesicle fusion, fusionmediated exocytotic neurotransmitter release, and whole cell fusion depending on the type of polar head group and on their relative proportions [20,23,24], as well as the susceptibility to respond to specific membrane or water-soluble fusogenic agents [25]. Other studies showed the importance of mutual thermodynamic-geometric compensations and lateral interactions among sphingolipids with different local geometries for controlling the curvature of self-assembled structures, depending on their relative proportions [26]. In addition, the amount of membrane fusion (and thus the release of nanovesicle content) can be controlled by a subtle variation of the relative proportions among a few fusogenic compounds, even if each of them individually facilitate HII-phase formation; this is due to thermodynamic-geometric compensations that can relieve or abolish membrane stress (Fig. 2) [23, 27].

In this work I will describe some representative results from over 40 years of research in our laboratory on local molecular properties and interactions determining supramolecular self-assembly, surface topography and structural topology that can mediate entrapping/release of bioactive compounds, membrane biocatalysis, and proliferation and differentiation of nerve cells grown on surfaces with a defined molecular organization.

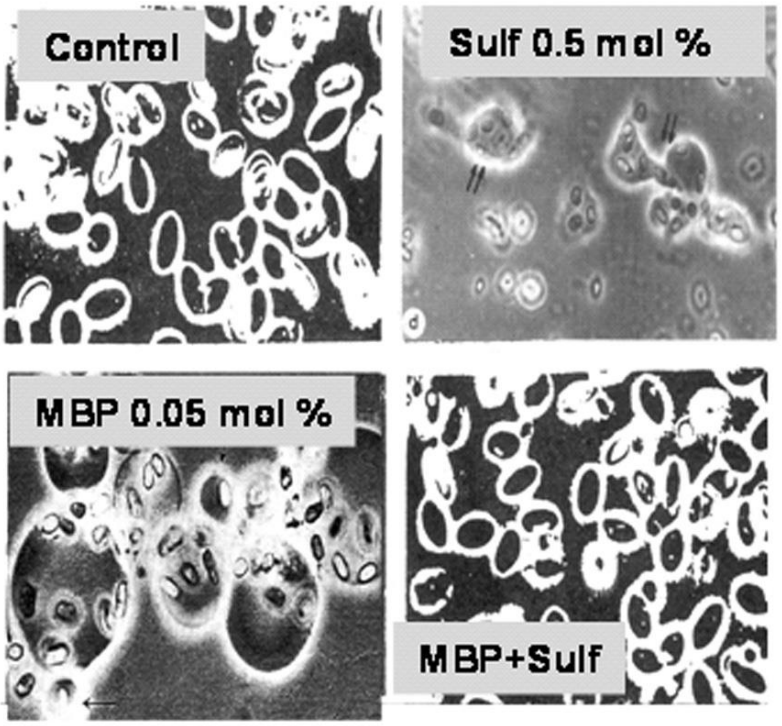

Fig. 2. Upper panel: cartoon showing coexistence of self-organized molecules and changes of supramolecular topology by compensated-uncompensated variations of molecular geometry; the center structure constitutes a base-morphology of a HII-phase within a lamellar phase. Lowe panel: formation of HII-phase is a pre-requisite for triggering membrane (and cell). Fusion/fission events. The photographs show control uninucleated chicken erythrocytes (upper left image), fused chicken erythrocytes chemically induced to form by incorporating the stated amount of sulfatide (upper right image) or myelin basic protein (lower right image) in the cell membrane; simultaneous incorporation of both fusogenic compounds results in lack of fusion (lower right image). See text and Maggio 1994)

Materials and Methods

A proper knowledge and control of themolecular parameters is necessary to understandthe multiplicity of synergic factors underlying the structural dynamics of biomembranes. The supramolecular/structural dimension spans scales from tens of nanometers to micrometers but itis at the "mesoscopic" level where the local molecularproperties become transduced (amplified,dampened or even abolished). The micrometer range, and above, includes structures and shapetransitions that can be visualized directly byconventional microscopy. The range of tens to hundreds of nanometers (the upper mesoscopic range) requires techniques with higher spatial and temporal resolution that exceeds the capacity of conventional microscopy because the structural dynamics begins to emerge and influences the behavior. On the other hand, at the lower mesoscopic range (nanometers to tens of nanometers) the highly dynamic molecular properties require more specialized techniques and modeling of the phenomena.

For the study and control at the different levels we employ some, or a combination, of the following techniques. For the lower mesoscopic range we can use high-sensitivity differential scanning (DSC) or isothermal titration (ITC) calorimetry, statistical thermodynamics of bi- and tri-dimensional phase transitions, measurement of average molecular parameters in monomolecular interfaces such as molecular surface density, surface free energy, bi-dimensional viscoelasticity, dipole potentials, real-time interfacial enzyme kinetics, surface application of local and external electrostatic fields, electron- and atomic forcemicrocopy, steady-state and time-resolved fluorescence spectroscopy, Fourier-transform

(FT-IR) and attenuated reflection (ATR) infrared spectroscopy, polarization-modulated absorbance/reflection infrared spectroscopy (PM-IRRAS), dynamic correlation light-scattering (DLS), nuclear magnetic (NMR) and electron paramagnetic (EPR) resonance spectroscopy, small (SAX) and wide (WAX) angle X-ray diffraction. For inspecting phenomena occurring on the upper mesoscopic range we have developed different techniques for reconstitution, under controlled molecular organization, of selforganized biomimetic lipid-protein systems with vectorial organization such as direct real-time visualization of the shape and dynamics of phase segregated domains of different composition along the lateral and transverse planes of biointerfaces. Their behavior can be inspected with epifluorescence microscopy, low- and high-resolution Brewster angle microscopy (BAM), imaging elipsometry and surface absorption/reflection spectroscopy using lipid-protein mono-and multi-molecular layers of defined single, binary, ternary, and more complex composition, ultra-thin-film transfer onto defined liquid and solid supports, immunolabeling, cyclic voltamperometry, perturbation with externally-applied electrostatic fields of controlled intensity, measurement of probe fluorescence to ascertain structural rearrangements with or without changes of topology, chemically-induced membrane fusion, fission, entrapment/release and targeting of bioactive compounds with lipidprotein reconstituted liposomes of defined composition and phase state [7-10, 28].

By proper combination of some or several of the techniques mentioned above applied to planar or curved interfaces one can know and control the mean molecular packing, the electrostatic dipole potential, surface compressibility and viscoelasticity, relative molecular orientation of reactive moieties, lateral and transverse surface topography of segregated domains and control of surface biocatalysis and structural topology.

Results and discussion

In the following section I will provide summarized highlights on how the chemicallyand physically-controlled organization of individual biomolecules and of their relative proportions allows to precisely specify their interactions, their miscibility properties and, hence, the surface topography and structural topology. The description will follow selected examples throughout more than four decades of work from our group on biomimetic self-assembed systems constituted by sphingolipids, synthetic amphiphiles, membraneactive proteins and phosphohydrolytic enzymes.

Control of lateral membrane organization

Through varied metabolically-driven, and relatively simple selective chemical changes that markedly alters the hydrophilic-hydrophobic balance and molecular shape, sphingolipids confer unique properties for information exchange in membrane dynamics. This represents an important key point at the mesoscopic level linking metabolism and self structuring, aided by the amplification capacity and transduction of surface tension represented by the reduction of dimensionality at the interface. In essence, such crosstalk begins at the very level of enzyme-driven surface catalysis whereby molecular structures are modified.

Fig. 3 summarizes the chemical structure of ceramide, sphingomyelin and more complex glycosphingolipids. The length of the Nlinked fatyl acyl chain in sphingolipids has a profound influence on its surface behavior [29]. The hydrocarbon moiety constituted by ceramide, an N-acylated sphingoid base, is a pivotal compound in sphingolipid metabolism and forms the biochemical basic stucture of all sphingolipids. In spite that short-chain ceramides are profusedly used to track or study the effects of “ceramide” in cell biology, a field that continues to attract scientist in the lipid signaling field, it is usually disregarded that ceramides

with different fatty acyl chains have dramatically different molecular properties. Surface pressure-molecular area isotherms of C14:0 and C16:0 ceramides indicated the formation of condensed films over all molecular areas. On the other hand, isotherms of C10:0 and C12:0 ceramides showed liquid expanded phases at large molecular areas and in-plane phase transitions to a condensed state at lower molecular areas. In the transition region, formation of flowerlike condensed phase domains occurr. The resultant perpendicular dipole moment of the monolayers calculated from the surface potential measurements and the film thickness, as determined by BAM, were shown to increase with the N-acyl chain length (Fig. 4). The overall results support the conclusion that the asymmetric portion of the sphingosine acyl chain can bend over the N-acyl chain in short chain asymmetric ceramides [30].

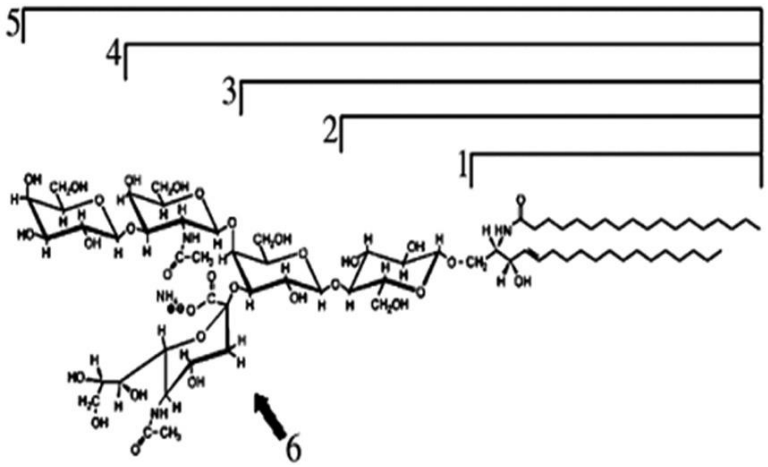

Fig. 3. Illustration of chemical structure of ganglioside GM1 [Galb1-4GalNAcb1-4Gal(3-2aNeuAc) b1-4Glcb1-1´Cer] and summary of neutral sphingolipids lacking the sialic acid residue NeuAc (6): Ceramide [N-acyl-sphingoid] (1), a monohexosylceramide [Glcb1-1´Cer] (2), a dihexosyl-ceramide [Galb1-4Glcb1-1´Cer] (3), a trihexosyl-ceramide [GalNAcb1-4Galb1-4Glcb1-1´Cer] (4), a tetrahexosylceramide [Galb1-4GalNAcb1-4Galb1-4Glcb1- 1´Cer] (5). Ganglioside GM3 corresponds to the structure of ganglioside GM1 but lacking the terminal disaccharide [Galb1-4GalNAc]; other complex gangliosides are constituted by addition of further moieties of sialic acid to the terminal galactose or to the sialic acid residues. For a structural description of a complete series of glycosphingolipids according to IUPAB rules see ref 6.

In the ceramide moiety, the presence of hydroxylated or non-hydroxylated fatty acyl chains induces variations of molecular packing areas, surface compressibility and phase state. However, a single carbohydrate residue in cerebrosides partially counteracts the surface effect induced by acyl-chain hydroxylation in the ceramide. This means that two defined changes of chemical structure become compensated in the supramolecular organization. Several other examples of these combined influences were described that can modulate the structural surface and bulk properties (see further refs. in [7-10].

On biochemical terms, biosynthesizing and degrading pathways of GSLs include specific glycosylating enzymes and glycosidases involving substrate-product transformations between GSLs leading to different oligosaccharide chain complexity. A variety of polar head groups, from a single hydroxyl group in ceramides to several neutral carbohydrates and sialic acid, confer particular charge, hydrogen bonding and hydration properties. The carbohydrates, linked by a- or b-glycosidic bonds form a neutral or negatively charged oligosaccharide chain protruding from the membrane perpendicular to the interface and into the aqueous medium contain a plethora of hydroxyl groups mediating stereospecific recognition to ions, lectins, toxins, enzymes, antibodies and other macromolecules [7-9]. Relatively small modifications of the sphingolipid chemical structure induce marked changes of molecular packing, phase state, surface electrostatics and membrane topology [9,30]. Since more than one surface parameter is simultaneously affected by the presence or absence of defined moieties the combined outcome is complex and difficult to predict.

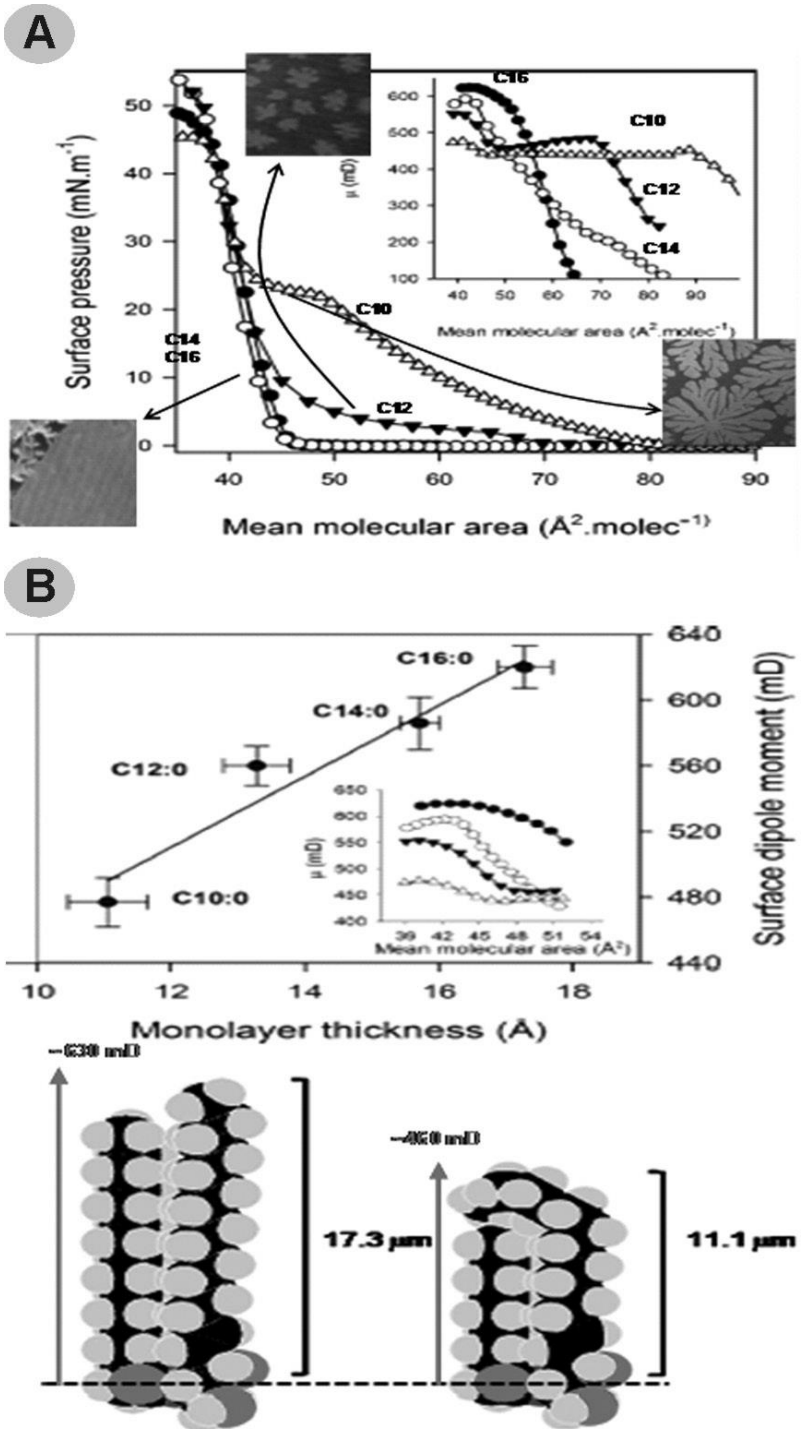

Fig. 4. A) Surface pressure-mean molecular area isotherms and dipole potential (inset) of Langmuir monolayer of ceramides. (●) C16:0 Cer, (○) C14:0 Cer, (▼) C12:0 Cer, and (▲) C10:0 Cer. Measurements were performed at 21oC. The photographs represent the typical liquid-condensed segregated domains in the region indicated by the arrows for each type of ceramide. B) *upper part*: Correlation between film thickness and maximum dipole moment of the conden sed phase ofceramides; Inset: resultant perpendicular dipole of the condensed phase of C10:0 Cer (△), C12:0 Cer (▼), C14:0 Cer (○), and C16:0 Cer (●). *Lower part*: molecular models generated with Alchemy III software (Tripos Ass.) of C16:0 Cer (A) and the proposed arrangement of C10:0 Cer (B). The scales indicate monolayer thickness measured by BAM, and the dashed line shows the probable location of the interface

A spontaneous chemical alteration in the oligosaccharide chain of possible physiological significance is ganglioside lactonization in which both the sialic acid charge and the ligosaccharide conformation are simultaneously and reversibly modified depending on the pH within the interfacial "physiological" range (for a bulk pH of 7 the interfacial pH can be more than 3 units below, depending on the surface charge density). This causes dramatic polar head group-dependent modifications of the sphingolipid molecular packing and dipole potential, further transduced to different interactions in mixed interfaces with phospholipids [32] without requiring slow biochemical alterations of the membrane composition. In the closely packed state there is a preferred orientation of the carbohydrate residues in the polar head groups, mostly perpendicular to the interface, which was confirmed with several methodologies (see further refs. in [7-10]). The orientation of the GSLs polar head group relative to the bilayer surface is not significantly influenced by the acyl chain chain length but it is sensitive to the lipid cross-sectional area and to the distance of the carbohydrates from the surface which may influence the conformation and orientation of the polar head group [6, 7], an important factor in ligand recognition.

Another major factor influencing membrane organization and dipole potential density is the structure and magnitude of the hydration shell associated to the polar head group. In addition, this is dependent on the long-range water structure in the bulk phase. Thus, hydrophilic solutes that modify entropy through changes of the water structural order greatly affect the surface organization. Interaction of acidic GSLs with proteins have also pointed out hydration-dehydration processes as having important influence on the intermolecular organization [7]. Studies with solvatochromic probes showed that the dye microenvironment at the interface becomes more polar (and more hydrated) in bilayers containing GSLs. When the two-dimensional order of the more complex GSLs is increased by compression, instead of water replacement, release of water molecules into the bulk phase occurs by coalescence of the GSLs hydration shell [7, 9].

Molecular self-miscibility and inherent phase state

It is increasingly more difficult to closely pack GSLs as they contain more complex oligosaccharide chains due to steric, dipole moment and electrostatic charge repulsion, as well as by hydration-dehydration effects mediated by the complexity of the polar head group, balanced by cohesive interactions among the hydrocarbon chains [7]. As a consequence, in a surface region where two different GSLs may coexist, the likelihood for lateral packing defects in the surface lattice increases with the polar head group complexity. When the oligosaccharide chain of the two GSLs is either relatively large or small, compared to the other, surface packing distortions and "molecular cavity" effects can occur whereby some molecules become "hidden" in terms of molecular area contribution, while affecting independently the surface electrostatics and lattice topography [9]. These effects are right in the range of translation of molecular-to-supramolecular phenomena regulating the surface miscibility among components.

It should be pointed out that the concept of "miscibility" can be quite ambiguous and misleading if not clearly focused on the particular scale range of the system to which it is applied [7]. For example, at the molecular level on the nm scale range intermolecular immiscibility in binary monolayers (with or without macroscopic phase domain separation on the mm scale range) can be inferred by additive variation of mean molecular area and dipole potential density as a function of the film composition, together with compositioninvariant collapse pressures remaining at the points of the lower- and higher-collapsing components [9]. In binary or ternary systems lateral immiscibility can be observed on the mm scale range by the presence of segregated phase orcompositional domains [10]. However, when the system is more

complex regarding compositionor surface topography the emergence of lateral thermodynamic tensions and interfacial energy terms eliminate cooperativity at the local (nm) level; this has the consequence of the film exhibiting additive behavior and smooth compression isotherms similar to that corresponding to a homogeneously mixed surface while it actually shows a richly featured surface topography with coexistence of immiscible domains of different composition and phase state [10]. Nevertheless, the existence of microheterogeneity on the mm scale range in the surface topography implies local interactions leading to favorable or unfavorable intermolecular mixing of the different components along the lateral plane on the nm scale range.

Similar to phospholipids, GSLs can exhibit all types of isothermal, surface pressureinduced two-dimensional phase states depending on temperature and the type of oligosaccharide chains [6, 9]. The more simple GSLs have mean molecular areas and polar head group sizes similar to those of phospahtidylcholines [6]. However, the values of the bilayer bulk transition temperatures (Tm) are 20-40oC higher compared to phospholipids with similar hydrocarbon hain length and unsaturation and this also occurs for sphingomyelin (see further refs. in [9]). Similar to the monolayer behavior, chemical or conformational differences in the oligosaccharide chain of sphingolipids introduce marked variations of the bulk phase transitions [7, 9]. A network of inter- and intra-molecular H-bonds involving the carbohydrates, the ceramide hydroxyl as well as the amide groups, together with the polar head group-bound water influences the interfacial properties and phase behavior.

The length of the N-linked fatty acyl chain has a profound effect on the phase state and domain morphology of ceramides [30]. As the number of carbohydrates (and also the possibilities for hydrogen bonding) increase in the oligosaccharide chain a decrease of Tm and transition enthalpy are observed, indicating diminished intermolecular cohesion. When the GSLs polar head groups are properly hydrated, extensive H-bonding to water increases the size of the lipid hydration shell and interferes with carbohydrate-carbohydrate interactions. This impediment can be further emphasized since the relative orientation of donor and acceptor groups at the surface, as well as the comparative sizes of the hydrocarbon portion in relation to interfacial curvature, are critical [6, 7]. The temperature required for reaching a fully liquid-expanded state in monolayers of GSLs decreases as the oligosaccharide chain becomes more complex and hydrated, similar to what is found for the gel-to-liquid crystalline bulk transition in aqueous dispersion [9].

Information transduction projecting molecular structure and lipid miscibility into segregation of membrane domains.

In most polymorphic liquid-crystalline systems of complex composition such as those constituted by biomembranes, the different unbalanced tensions result in lateral or transverse segregation of components. Biomembrane surfaces are loosely ordered microheterogenous structures. These are in a metastable far-fromequilibrium, state in which thermodynamic, geometrical and viscoelastic tensions determine the spontaneous emergence, shape and composition of segregated domains as transient patches of different composition and/or phase state.

The idea of compositional or phase domain segregation, well known to membrane biophysicist for over 40 years, simply reflects the basic physico-chemical phenomena of eliminating or reducing local tensions derived from thermodynamic incompatibility among different molecules by establishing lateral or transverse immiscibility, a symmetry-breaking event with long-range supramolecular consequences [14]. Another important finding in simple

binary systems was the description of the in-plane coexistence of two phases in the liquid state but with different organization, the liquid-disordered and liquid-ordered phases, with the important influence of cholesterol in the latter. Liquid-liquid phase coexistence in whole natural membrane surfaces with a complex composition was later demonstrated [9, 10].

Close molecular packing among relatively complex sphingolipids is thermodynamically unfavorable at the local level and they become expanded in proportion to the complexity of the oligosaccharide chain [6-9]. The type and relative complexity of the polar head group of a natural sphingolipid species in a binary mixture are of paramount importance for establishing the molecular miscibility properties, the type, and the particular details of the interactions. Interactions among neutral GSLs, and of the latter with ceramide, occur with hermodynamically unfavorable expansions of the mean molecular area and increases (hyperpolarization) of the resultant molecular dipole perpendicular to the interface.

In binary mixtures of ceramide with neutral GSLs the changes are more marked when the oligosaccharide chain is more complex; the intermolecular incompatibility is further emphasized when the binary mixture is constituted by two neutral GSLs with similar polar head group sizes containing an increasing number of carbohydrate residues. On the other hand, interactions of ceramide with gangliosides are characterized by thermodynamically favorable condensation of the mean molecular area (reflecting increased intermolecular cohesion) and favorable matching of the resultant molecular dipoles leading to surface depolarization, in proportion to the complexity of the ganglioside polar head group. All mixtures between different gangliosides reveal immiscible behavior on the molecular (nm) scale range. Based on local average molecular interactions it was concluded that it is thermodynamically unfavorable for GSLs with polar head groups of similar size to undergo mixing between them [34].

Differences in length (chain asymmetry) of the sphingosine base and the N-linked fatty acyl chain in ceramides is of paramount importance for determining their miscibility, phase segregation and domain thickness among different ceramides (Fig. 5). This is also reflected on the surface (dipole) potential of the interface as a whole. The capability of even a single type of ceramide to induce formation of solid-like segregated domains, with effects on the more liquid-like regions of membranes, should be considered only by strictly defining the type of Nlinked acyl chain composition of the ceramides involved and how the components mix among themselves. Actually, one of the most commonly found ceramides in biological systems, C16:0 Cer is not completely solid, contrary to what is usually conceived, but displays a rich phase coexistence even at physiological temperatures; with shorter chain expanded lipids it can also form expanded mixed phases depending on the surface pressure [35]. Thus, transient mixing–demixing processes among different ceramide species can occur in biomembranes, both inside ceramide-enriched rigid domains or platforms as well as in the expanded phase which can regulate lateral partitioning of lipids and proteins.

On the molecular (nm scale) range all GSLs and ceramide were found to spontaneously mix non-ideally in monomolecular films with different phospholipids [6, 7]. In bulk dispersions, the temperature-composition phase diagram of binary systems of DPPC and neutral GSLs with different oligosaccharide chains show marked gel-phase and some liquid-phase immiscibility in which isothermal melting of the pure phospholipid is observed over a rather wide range of composition; however, no isothermal melting corresponding to laterally segregated pure GSLs is found over the whole phase diagram and not even on the GSL-rich side of the composition range. This behavior was pointed out many years ago (see refs. in [6]) as clearly indicating that GSLs do not tend to form a separate phase by themselves but actually become segregated in GSLs-enriched, but mixed, clusters with phospholipids, driven by the spontaneous phase separation of isothermal melting pure phospholipid domains that exclude GSLs [9]. In fact,

interactions among neutral GSLs are too short-range (mostly different types of H-bonding) to constitute a driving force for GSLs clustering and the negative charge on gangliosides precludes their close association. Intermolecular H-bonding, clearly demonstrated only for the more condensed GSLs (cerebrosides) do occur, and may help stabilize specific GSLs within a localized membrane region but only after or during domain formation driven by longer range thermodynamic potentials [8, 9].

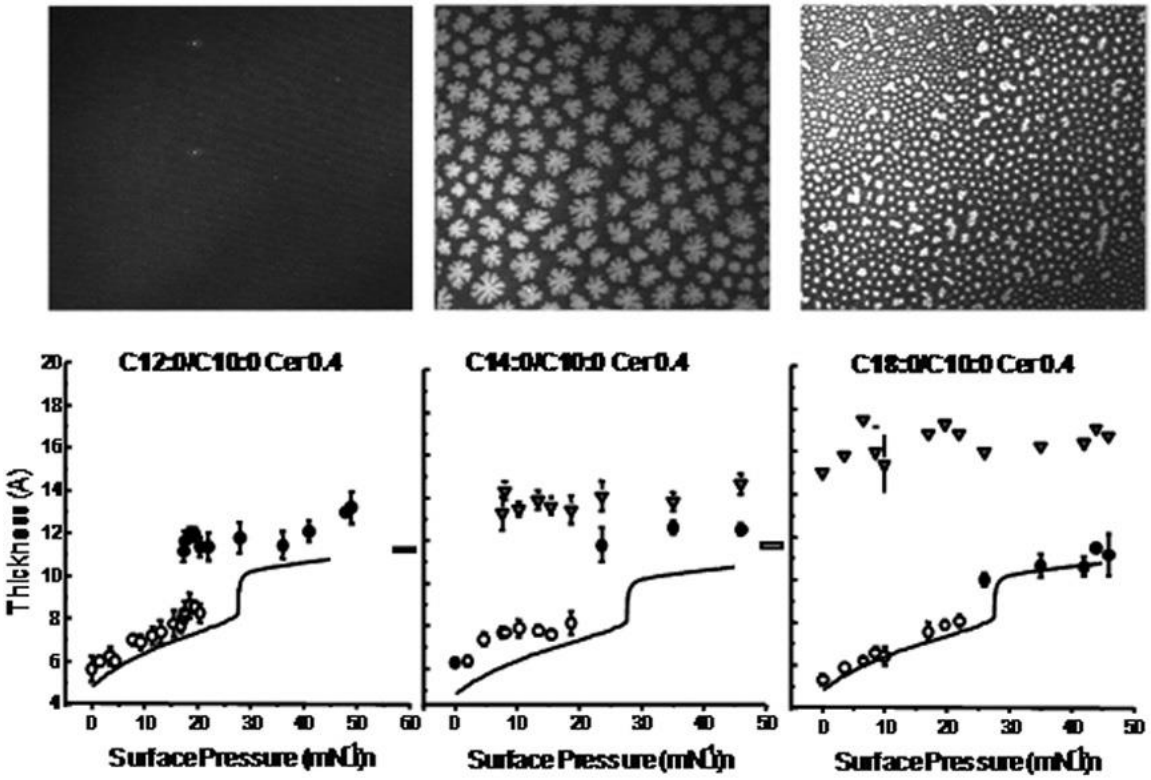

Fig. 5. Representative behavior of miscibility among asymmetric ceramides. The upper panels show the surface topography (BAM) of mixed films of the ceramides in the mole fractions indicated (0.4 for the ceramide with the longer N-acyl chain) ; the lower panels show the variations with the surface pressure of the optical thickness of the liquid expanded (○) and condensed (●) phases, together with the coresponding thickness variation for pure films of C10:0Cer (continuous line) as it undergoes the transition from the liquid-expanded to condensed state. Full (C12:0/ C10:0 Cer) , partial miscibility (C14:0/C10:0 Cer) or immiscibility (C18:0/C10:0 Cer) of the C10:0 Cer, depending on the N-acyl chain length of the other ceramide can be clearly ascertained.

Membrane proteins can stabilize and retain defined gangliosides (such as GM1) and anionic (i.e. sulfatides) or neutral (i.e. cerebrosides) GSLs in compositionally-segregated surface domains, as recently found in monolayers formed with all the lipid and protein components of whole myelin membrane (Rosetti et al., 2008). Unlike epifluorescence that distinguishes the differential partitioning of fluorescent probes, Brewster Angle Microscopy derives contrast from differences in the optical properties of thin films [36] and does not require a reporter probe to ascertain phase coexistence. Through a quantitative measurement of the light reflected at the interface, BAM also allows to calculate the relative change in optical thickness of defined surface regions. The pattern observed by BAM indicates that segregated domains have a different optical thickness and showed a transition from round-border domains to fractal domains occurring during compression [36].

Another type of glycolipid results from the modification of Cyclodextrins (CDs) with acyl chains. These are macrocyclic oligosaccharides capable of forming reversible non covalent complexes with a wide variety of guests, which allows them to function as all-purpose molecular containers [37]. The host-guest complex formation provides to the guest a different microenvironment that changes the physico-chemical properties of the included molecule such as its chemical reactivity, stability, solubility and spectroscopic properties (see refs. in [37]). Because of that, important applications were found for their native form as well as for some derivatives.

Modified CDs were successfully applied in constructing molecular carriers and platforms with important perspectives for nano-applications (see refs. in [37]). For a successful design and application of CDs self-assemblies, a fundamental understanding of their self-structuring is crucial in order to provide a solid basis for their rational use in nano-construction. Different CDs derivatives have been prepared by substituting their primary or secondary hydroxyl groups thus obtaining highly amphiphilic compounds. The full substitution of cyclodextrins leads to rigid cylindrical structures that are very anisotropic regarding the vectorial separation of the hydrophobic and hydrophilic moieties. This confers to these molecules a high stability when organized in monolayers at the air-aqueous interface. However, full substitution also confers scarce conformational flexibility to the CD ring and therefore little possibilities for adopting different intermolecular arrangements in the monomolecular film [37].

The presence of a single hydrocarbon chain linked to one glucopyranose unit in the cyclodextrin ring brings about more possibilities for different organizations depending on the lateral surface pressure. The films formed by the amphiphilic cyclodextrin C16-ßCD (Fig. 6) show distinctive structural feature such as variations of the surface packing, dipole moment, surface topography and packing-dependent reorientation of the ßCD ring. Also, it was found that the organization adopted by the film showed surface pressure-dependent packing hysteresis with the consequent potential for structural information storage [37]. The specific orientation acquired by the oligosaccharide ring of C16-ßCD was more precisely inspected by using the variation of the PM-IRRAS band intensity ratios corresponding to specific vibrations of the cyclodextrin moiety [38]. The selection rules of PM-IRRAS spectroscopy at the air-water interface allow determining the orientation of the transition moments of vibrational modes relative to the interface. The up (down) sense of a band relative to the baseline and its intensity are dependent on the orientation of the corresponding transition. If the transition moment is parallel to the interface the absorption results in a strong positive band, whereas the band is negative if the transition moment is perpendicular to the surface. Between these two extreme cases an intermediate orientation of the transition moment will led to compensated positive and negative contributions thus affecting specific band relationships. Detailed structural data referred to the reorientation and intermolecular interaction of amphiphilic cyclodextrin in self-organized monolayers at the air-water interface can be obtained by PM-IRRAS analysis. Furthermore, with this technique it is possible to disclose the presence of intermolecular hydrogen bonding network among ßCDs rings [38].

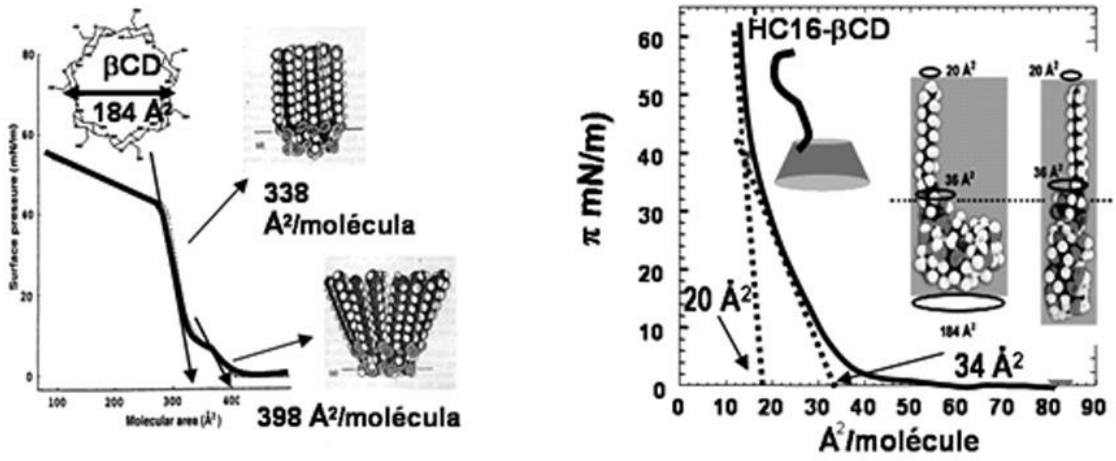

Fig. 6. Surface pressure-mean molecular area compression isotherms of acylated- b-cyclodextrins. Left panel: per-acylated cyclodextrins, the scheme and cartoons indicate the cavity zize and the limiting cross-sectional mean molecular areas in the liquid-expanded and condensed states (see arrows). Right panel: mono-acylated b-cyclodextrin indicating the limiting cros-sectional mean molecular areas (dashed lines with arrows) of the liquid expanded and condensed states; the upper cartoons shows the b-cyclodextrin cavity oriented parallel to the interface; the molecular models represent the mono-acylated b-cyclodextrin with the cavity oriented parallel (left model) and perpendicular (right model) to the interface, depending on the lateral surface pressure; the left model can not be justified by the limiting cross-sectional mean molecular areas experimentally determined at the interface while the right model is compatible with the compression isotherm.

The effects described in the previous section clearly represent an extraordinary capacity for lipid-protein information transduction that is conveyed laterally on the surface but also across the membrane plane. The different length and/or average orientation of the oligosaccharide chains present in GSLs existing in various phase states clearly suggest a relationship between the type of polar head group and the lateral topography and interfacial thickness.

Sphingolipids follow different regimes of variation of reflectance versus surface pressure according to their phase state. Cer and GalCer, with polar head groups containing only a hydroxyl group or a single galactose residue, undergo small changes of reflectance indicating that, on average, the film thickness remains rather constant during compression [36]. These results are in agreement with previous ones showing that these lipids form very condensed films, with small changes of intermolecular packing and polar head group orientation under compression [9]. The topographic appearance by BAM of films of GalCer and Cer at low surface pressure is dominated by the presence of highly mobile, rigid cluster domains, with irregular boundaries, coexisting with gas phase. As the film is compressed, the clusters fill out the optical field and the surface acquires a more homogeneous appearance. Asialo-GM1 (Gg4Cer) and tge mono-sialoganglioside GM1 have large polar head groups constituted by oligosaccharide chains of similar lengths, with four neutral carbohydrate units in Asialo-GM1 and one negatively charged sialosyl residue in the ganglioside. These results in a liquid-expanded interface and BAM images reveal a rather homogeneous surface with the reflectance showing notorious changes during compression [36]. This agrees with previously published measurements of surface (dipole) potential that indicated reorientation of the oligosaccharide chain of both lipids depending on the surface pressure [6-10].

The perpendicular dipole moment of Cer increases as the molecules become more closely packed due to the surface pressure-induced stretching of the hydrocarbon chains as they become condensed. It is well known that the positive end of the dipole moment of aliphatic chains points up to the air side of the monolayer; for a saturated chain of 16-18 carbons the magnitude of the dipole in the condensed state amounts to a maximum of about 350 mD [6, 7, 36]. The maximum

achieved by Cer reaches 562 mD due to the influences of some unsaturation and the hydrated hydroxyl group in the polar head. The thickness of interfaces formed by ceramides with increasing hydrocarbon chain asymmetry is linearly related to the dipole potential and indicated the adoption of a particular structural kink of the N-linked fatty acyl chain when the asymmetry is high [30]. The carbohydrate residues of GSLs contribute with a resultant dipole moment generally oriented opposite to that of the hydrocarbon portion and increases with the complexity of the oligosaccharide chain [6] which brings about a decrease of the molecular dipole moment. In addition, the variations with packing of the resultant dipole moment of the GSLs indicate oligosaccharide chain reorientation into the aqueous subphase more perpendicular to the interface. In good agreement with these results, the surface reflectance increases gradually with film compressions suggesting progressive thickening of the interface [36].

The polar head group size, protrusion and optimal area exposed to the aqueous phase in relation to the volume and length of the hydrocarbon moiety determines a molecular shape further away from a cylinder and more similar to a cone as the GSLs are more complex [16]. Theoretical and experimental studies have shown that neutral GSLs are compatible with a bilayer structure of increasing curvature in the order GlcCer ≈ Gl- Cer > LacC > Gg3Cer > Gg4C. For GSLs with relatively short oligosaccharide chain the packing constraints allow their presence in relatively stable bilayer vesicles, with a low free energy per molecule and the competing factor limiting their size being the entropy of the ensemble [16]. This imposes an upper limit for the number of lipids forming the structure that must be compatible with the entropy of the aqueous phase that continuously favors maximal lipid packing in order to minimize the lipid/hydrocarbon interface.

Positive interfacial curvature is favored due to the increase of area per polar head group exposed to water in relation to the hydrocarbon chain volume; on the other hand, a similar effect would occur at constant lateral surface pressure for the more complex GSLs because of the increased molecular area required by the hydrated oligosaccharide chain [16]. Thus, factors that affect intermolecular packing are simultaneously transduced to curvature alterations of the interface and vice-versa, with the surface free energy of the molecule varying in correspondence; within certain limits this may not conflict with entropy but as the molecular geometry supports increasing stress the structural stability will be affected with the consequence of amplifying the lateral perturbations influencing surface topography changes and/or topological rearrangement. Domains enriched in complex GSLs should spon-taneously tend to increase curvature away from the aqueous interface. These effects will occur spontaneously whenever lateral pressure luctuations drive the molecular packing areas to exceed the critical limits compatible with the interfacial curvature.

Two major consequences may occur depending on how the stress is transduced by relaxation. If the fluctuation is relatively small it may be absorbed by the membrane elasticity; the periodical temporal and spatial tangential stress wave and variation of compressibility may dissipate through other factors that control surface topography such as changes of the phase state and intermolecular interactions in both the polar head group and hydrocarbon regions [7-9,13]. If the magnitude of the fluctuations is such that the stress cannot be relieved, the membrane curvature will have to concede changes inevitably introducing tension energy costs; this may be balanced or contained up to when the membrane elasticity becomes no longer compatible with the surface stress at which point the aggregate undergoes abrupt reorganization in a different structural topology [16]. There are many experimental observations confirming early findings [6, 9,16] regarding the combined and amplified influences of the local conformation, overall hydration, charge, size and orientation of the

oligosaccharide chain in determining the thermodynamic stability, shape and lateral topography of GSLs in selfassembled interfaces.

Thermodynamic-geometric compensations and topology

In systems constituted by more than one type of molecules, there may be further compensations that enhance or alleviate stress, reflected in the overall topology, besides the intramolecular balance of factors transduced to lateral and transverse tensions. It was shown that small amounts of HI- phase-forming lipids such as specific gangliosides in binary or ternary mixtures with other lipids that spontaneously tend to form non-bilayer HII-phase cause facilitation, impairment or elimination of the HII-phase structure [17], depending on their relative proportions, a finding also confirmed using other HII-phaseforming lipid mixtures [18].

In GSLs the sphingosine base of 18 carbons penetrates into the bilayer to a depth of only about 13-14 carbons while the amide-linked fatty acyl chain is even longer than the hydrocarbon portion of most phospholipids and can extend to a length of 20-24 carbons. It was thoroughly demonstrated that chain disparity leads to chain interdigitation with the important implication for transversal information transmission by hemibilayer coupling [14]. The long-chain fatty acyl residue of some GSLs extends across the lipid bilayer mid plane and penetrates substantially into the opposing monolayer. When the proportion of asymmetric sphingolipid is increased above 30 mol % the membrane adopts a partially interdigitated structure depending on composition and temperature. If the hydrocarbon chains of ceramide, the basic moiety of all GSLs, are sufficiently asymmetric it can undergo chain interdigitation in phase separated ceramide-enriched domain depending on the relative proportions with phospholipids [26, 39]. This inherently implies the capacity for transverse information transduction since both halves of the bilayer become essentially coupled in those membrane-spanning regions.

Another manner for transmitting transverse information across the membrane is by topological rearrangement involving non-bilayer phases. Ceramide has a very small polar head group in relation to the hydrocarbon chain volume which conveys a preference for self-organizing into negative curvature structures, favoring HII-type of phases on which basis it can flip-flop relatively fast across the bilayer (see refs. in [40]). In addition, hexagonal-II phase like zones are key structural intermediates for inducing cell and lipid bilayer membrane fusion or fission [21]. The interference of GSLs with HII-phase formation was demonstrated for a mixture of total bovine brain gangliosides, GalCer and cerebroside-sulfate in correlation with the long known capacity of several GSLs to affect cell [7] and bilayer vesicle fusion induced by various fusogenic agents [18, 24].

Transient structures of the HII-phase type are important intermediates involved in the hemi-fusion and whole fusion of membranes [21] that can be triggered by a variety of lipids [20] and proteins [27]. Gangliosides and other GSLs can induce or inhibit cell fusion, hemi-fusion and whole bilayer vesicle fusion depending on their relative proportions with respect to phospholipids and the type of their oligosaccharide chain [24] an effect fully consistent with the distortion of geometrical shape and tension discussed above (see Fig. 2). Thermodynamic-geometric compensations can abolish membrane stress leading to fusion when two fusogenic compounds, each of them individually facilitating HII-phase formation, are simultaneously present in the membrane [23, 26, 27].

Self-structured lipid-protein topography and manifold control of surface biocatalysis

As a general overall concept, it is just thermodynamically inevitable that at least one way of relieving lateral and transverse tensions is by segregation of immiscible components into separate domains of different composition and/ or phase state. However, it has been much moredifficult to describe the local molecular properties and defined interactions representing the critical thresholds driving the intermolecular immiscibility processes leading to domain formation on the mesoscopic level.

In this section I will summarize selected examples of the involvement of extrinsic or integral proteins, and a membrane-active phosphohydrolytic enzyme, in the process of lateral segregation of phase domains, sculpturing of the surface topography, and the bi-directional modulation of its biochemical and structural reactivity. The description will be restricted to some surface effects of myelin basic protein, Folch-Less proteolipid protein and a neutral sphingomyelinase.

Thermodynamic domains induced by MBP in lipid mixtures of myelin glycosphingolipids

Apart from preferential penetration into, and interactions with, negatively charged interfaces containing sulfatides and gangliosides, MBP causes lateral condensation and affects differentially the thermotropic behavior of single glycosphingolipids (see refs. in [7, 10]). In ternary systems of MBP in bilayers of defined composition constituted by DPPC and different glycosphingolipids the protein causes phase separation and can induce membrane-membrane interactions and recombination depending on the oligosaccharide chain of the glycosphingolipid [8-10]. The protein affects the thermotropic behavior over both the short- and the long-range regarding the features of the segregated phase domains.

Even in simple mixtures of DPPC with the neutral galactosylceramide the protein causes a major asymmetric distortion of the phase transition; this indicates that MBP preferably partitions into the liquid-crystalline lipid phase in which the high temperature asymmetry can be resolved into a broad low-cooperativity peak. Statistical thermodynamic calculations of the cluster size distribution functions reveal that the most probable number of thermodynamicallycorrelated molecules undergoing the transition at T½ forming the high temperature clusters induced by MBP is below 40 and is narrowly distributed. On the other hand, the cluster size and distribution of the segregated domains undergoing the phase transition at the T½ of the protein-free mixture remain practically unmodified [8, 9]. In binary lipid mixtures of DPPC with sulfatide and ganglioside GM1 (two major anionic sphingolipids of myelin) MBP induces major changes of the thermotropic behavior. Previous publications reported the complete temperature-composition phase diagrams for binary mixtures of several glycosphingolipids with DPPC see refs. in [7]. Those studies revealed that for protein-free mixtures containing sulfatide and gangliosides the phase diagrams were quite broad and, depending on composition, phase coexistence of phospholipid domains excluding glycosphingolipid-enriched domains were present. The presence of MBP in increasing amounts facilitates the formation of the high-temperature segregated glycosphingolipid- enriched domains.

Sphingomyelinase-mediated surface sculpturing

SMase appears to be sensitive to the phase state of the substrate-membrane. Several studies both in lipid monolayers and bilayers showed an enhanced activity of SMase when the substrate is in a fluid state compared to the gel state. It was also described that SMase is modulated by the presence of a liquid-ordered, *lo* (cholesterol-enriched), phase showing

increasing activity in the order gel<*lo*<fluid phase [41]. For the largely studied reaction catalyzed by phospholipase A2 (PLA2) several studies have related "membrane defects", such as those arising from the coexistence of lipid domains in different physical state, with enhanced phospholipases catalytic activity. A proposal was made that the LE-liquid condensed (LC) lateral interfaces (lipid domain borders) in a one component monolayer of dipalmitoylphosphocholine (DPPC) acted as starting points for PLA2 catalytic activity (see refs. In [41]); however, evidence also exists indicating that the surface mixing of the lipid substrate with the products of the enzymatic reaction can account for the surface topography, even in enzyme-free systems (De Tullio & Maggio, to be published).

For SMase, the presence of Cer-enriched domains in the monolayer prior to the SMase action on SM/Cer monolayers favors the activation steps in a way that modulates the morphology of domains generated by the enzyme action and is dependent on the amount of the lateral interface present [15]. These results supported the strong (and generally accepted) hypothesis that lipolytic enzymes depend on physical contact at the domain boundaries in order to become fully active. This "perimeter-activated mechanism" requires revision based on several recent studies.

Recently, we proposed a mechanism on the basis of the area-activated hypothesis for the enzymatic action of SMase that can also explain the regulatory effect of the surface pattern. SMase adsorbs homogeneously to the LE interface and exerts its catalytic activity promoting homogeneous enzymatic generation of Cer in the LE phase. Cer is partially immiscible in the SM-enriched phase [41]. When SMase acts on a uniform LE phase, the rapid production of Cer leads to a metastable, kinetically trapped mixed monolayer, that becomes supersaturated with the latter lipid. This effect acts as driving force for the segregation of Cer-enriched domains following classical nucleation mechanisms. The nucleation process involves a kinetic barrier with consequences on the lengthening of the lag period before full enzymatic catalysis. Accordingly, the number and size of Cer-enriched domains are determined by the extent of Cer oversaturation in the LE phase rather than by the SMase local activity (Fig. 7bd). When SMase exerts its action on a lipid interface that initially shows phase coexistence, the newly formed Cer molecules rapidly diffuse from the LE phase to incorporate into the Cer-enriched (pre-existent) domains. This last situation eludes Cer oversaturation in the SM-enriched phase and therefore, domain nucleation [41, 42].

Supporting such proposed mechanism, it was found [41] that SMase-treated SM/Cer monolayers rather show growing of pre-existing domains than nucleation of new domains. As a consequence, product inhibition of the enzyme is reduced and the pre-catalytic steps can be overcome in a shorter time. In this manner the presence of Cer-enriched domains, micrometers away from the enzyme location, can regulate the enzyme action at a long-range. SMase catalytic activity is favored by the clearance of the product Cer from the LE (substrate) phase that diffuses to incorporate into the Cer-enriched domains. This effect promotes a low product concentration in the enzyme environment as far as Cer diffusion is fast enough, compared to the Cer-production rate, for an efficient SM/Cer demixing. This hypothesis is consistent with the finding that SMase show less activity when acting on more condensed phases (LC/gel phases or cholesterol containing phases) where Cer diffusion may be restricted (see refs. in [41, 42]).

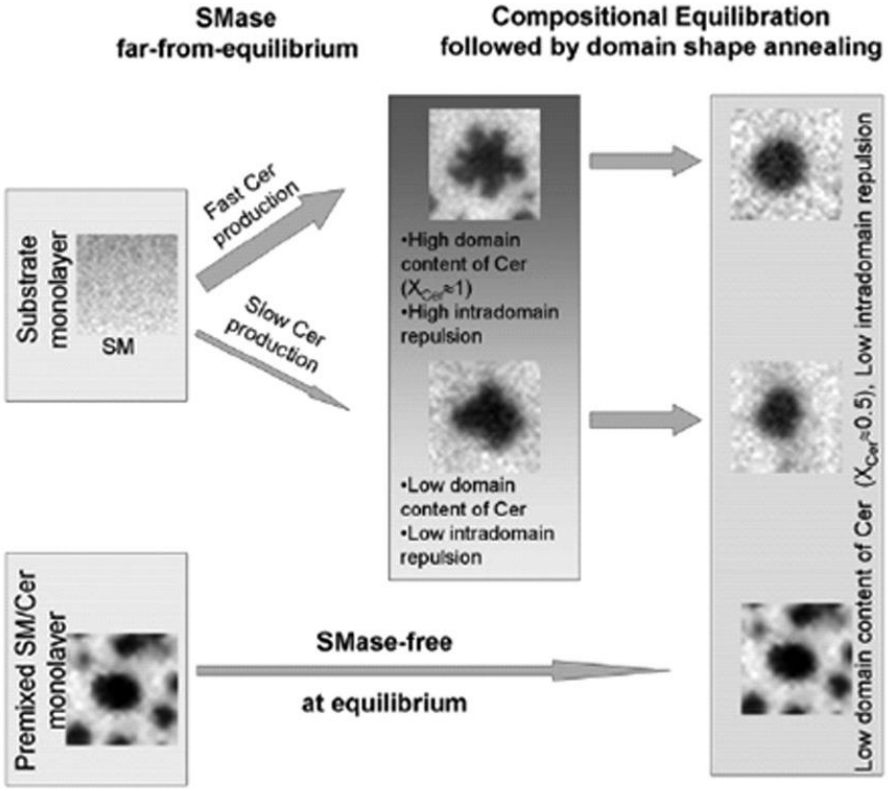

Fig. 7. The schematic representation summarizes domain evolution in a SMase-driven or in an enzyme free SM/Cer interface, depending on the rate of ceramide formation in a far-off equilibrium (high enzyme rate), near-equilibrium (moderate or low enzyme rates) or in-equilibrium (very slow enzyme rate or pre-mixed enzyme-free films).

Domain shape rearrangement following out-of-equilibrium compositional change by SMase action

Active and functional membranes are far-from-equilibrium structures and evolve in steady state conditions controlled by fluxes of energy and matter. Consequently, thermodynamic non-equilibrium effects have direct relevance for the lateral organization of membrane components. A relatively rapid enzymatic generation (< 15 min) of Cer by SMase in SM monolayers leads to a fundamentally different surface morphology and pattern organization when compared to enzyme-free premixed SM/Cer monolayers of the same lipid composition [41]. Detailed pattern analysis by image processing techniques revealed substantial differences in both systems: i) domains formed by the action of SMase show regular sized star-like shapes while condensed rounded domains are observed in the enzymefree films; ii) in the SMase treated monolayer the interdomain energies force the Cer-enriched domains to adopt a predominantly hexagonal lattice, while less ordered lattices were observed in enzyme-free SM/Cer monolayers, and iii) only in the case of the enzyme-free SM/Cer monolayers, the LC domains formed cover an area whose relatively large size cannot be accounted for by a phase of pure Cer. These differences appear to be the consequence of an out-of-equilibrium state of the SMase coupled monolayers (Fig.7). In consequence, the surface "stores" selective structural information depending on its genesis that is subsequently transduced to the surface topography [41, 42].

The cross-talk between lateral domain structures, dipolar electrostatic fields within and outside the segregated domains, and the effects on biocatalysis add new and rich perspectives for the mechanisms of phospholipase-mediated signal transduction in biological

membranes. Also, surface-mediated cross-talk between SMase and PLA2 activity in monolayers, with the involvement of the respective lipid substrate and products, has been previously described (see refs. In [41, 42]).

Physical control of enzyme activity and topography by electrostatic fields

The application of electrostatic fields of defined strength and polarity can control hosphohydrolytic reactions at the interface by reversibly activate or inhibit PLA2 activity [43]. The effects of several sphingolipids on PLA2 activity is significantly correlated to their dipolar and packing properties [9]. PLA2 activity against mixed lipid monolayers of phospholipids and sphingolipids can be directly modulated by the application of constant electrostatic fields in relation to their magnitude and polarity. In addition, the effects of the external field are superimposed onto and modulated by the depolarization or hyperpolarization induced at the interface by the specific dipolar properties of the sphingolipid molecules in the mixed films. Hyperpolarizing fields enhance the enzymatic activity against pure dilauroylphosphatidic acid while depolarizing fields induce a decrease of activity. Compared to the pure substrate, when the interface containing other lipids is already partially depolarized the magnitude of activation induced by an external hyperpolarizing field is decreased; conversely, depolarizing fields cause an increased inhibition of activity on substrate interfaces that are initially depolarized in part by the presence of other lipids. The effects of sphingolipids depend on their relative proportion in the monolayer. In summary, the activity of PLA2, in addition to responding reversibly to the direct application of external electrostatic fields, is modulated locally by the polarity and magnitude of the lipid polar head group dipole moment [43].

Lipid and protein molecules anisotropically oriented at a hydrocarbon-aqueous interface such as that existing in biomembranes configure a dynamic array of self-organized molecular dipoles. These can act as sensitive local and longrange sensors of the electrical properties along and across the membrane interface. Intrinsic electrostatic features, intermolecular packing and interactions determine a resultant dipole moment density that in conjunction with line tension forces, in the case of molecular immiscibility, are major factors responsible for the individual morphology of coexisting phase domains as well as their lattice organization along the surface [10, 28, 44, 45].

Externally applied electrostatic fields induce a separation of previously existing phases in a monolayer and cause phase separation in an initially microscopically homogeneous system in the absence of the applied field [44, 45]. In a recent work, it was demonstrated the importance of the dipole moment density difference among the immiscible sphingomyelin and ceramide monolayer, coupled to the domain boundary line tension, as major factors to establish both the characteristic domain morphology and their lattice organization in premixed binary films and in those generated by the action of sphingomyelinase [41]. Cer-enriched domains in a Sm-enriched media behave like other mixtures in the sense that positive potentials applied to the upper electrode reversibly repel the domains and negative potentials attract them, but at 10 mN m-1 a threshold potential is required for an increase of domain displacement. The observed field effects strongly depend on the lateral pressure. None of the analyzed parameters that describe the domain properties show significant changes with the lateral pressure. A resultant dipole moment component of the domain that is parallel to the interface and is dependent on pressure is suggested by the induced electrostatic response of the lateral domain topography. The monolayer viscosity appears as a major factor that modifies the field effect in a surface pressure-dependent manner [44].

Biocompatible self-organized ultrathin films from whole cell membranes

Self-organized surfaces can be formed with whole natural membranes with all their compositional complexity. The first microscopic observation of a freshly prepared monolayer from a natural membrane [46], containing all the components of whole myelin, indicated a rich and complex heterogeneity along the lateral plane (Fig. 8). Coexistence of domains in at least two major liquid phases was first described in myelin monolayers and a subsequent similar approach using kidney brush border membranes also showed liquid-liquid phase coexistence (for refs. see [36]). The super-structuring of the myelin monolayer (i. e. shape and size domain distribution), changed upon compression from one morphology dominated by rounded liquidexpanded domains (equilibrium radius $Re \approx 70$ µm) at low surface pressure to fractal domains formed at higher surface pressures in a selfsimilar topography lacking a characteristic size (fractal dimension $Df \approx 1.7$). The change from a circular pattern to a fractal one does not show a clear transition point and occurs over a range of 15-30 mN/m (for refs. see [36]) .

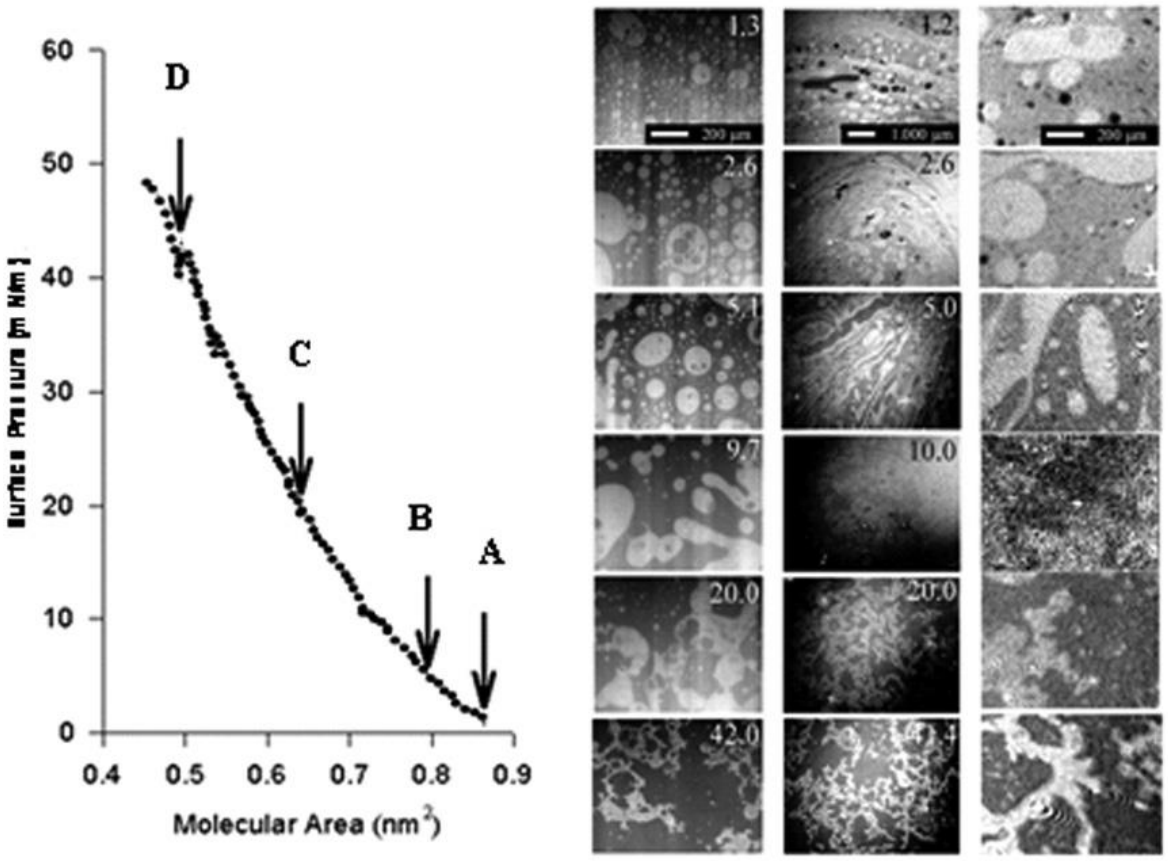

Fig. 8. Left plot: surface pressure-mean molecular area compression isotherm of a monolayer formed with the whole myelin membrane at the air-water interface. Right photographs: images of the surface topography taken at the surface pressures indicated with letters on the isotherm. The first image column from the left corresponds to monolayers doped with 1 mol % NBD-phasphatidylethanolamine to visualize liquid-expanded domains (bright) and liquid-ordered domains (dark); second and third image columns are Brewster Angle Microscopy (probe free) visualizations of the surface, at similar surface pressures, at two different magnifications (see scale bars) showing thicker (bright) and thinner domains (dark).

The myelin monolayers can be transferred onto sylanized glass and immuno-labeled, resulting in a map of the component labeling distribution. This showed two phases in which the labeled components remain within the same domain between 12 and 40 mN/m. Thus, although the distribution and shape of the domains changes upon compression, the composition of the phases appears to remain unaltered, at least qualitatively [36].

The precise control and knowledge of the surface topography formed by whole nerve membranes, or with self-organized reconstituted interfaces formed by some of its purified components, provides a fundamental platform for constructing supports onto which neural cells can be cultured. The contact, proliferation and differentiation response of such cells depends on favorable stimuli provided by different trophic factors from the surrounding medium and from the contacting surface that are sensed by the cell plasma membrane. In this manner, by proper control of the surface composition and organization, the cell growth can be directed and controlled. Pioneering experiments along this line were initiated over the last 20 years by first forming monolayers of axolemma membranes under strict control of its molecular organization, and their transfer on solid supports onto which Schwann cells were subsequently cultured. The growth pattern of the cells depended selectively on the lateral packing and surface electrostatics and responded by changing accordingly their proliferation, differentiation and exposure of lipid and protein membrane antigens [47, 48].

The preparation of the first monomolecular layer of defined composition, thickness and phase state of a glial membrane such aswhole myelin was also achieved and it was shown that it conserves many structural features of the natural membrane (for refs. see [36]). These preparations provide natural, self-organized and biocompatible surfaces bearing well controlled/ directed molecular organization that can be used as cellular supports in view of the neuron-glia cross-talk for axonal guiding and targeting along directed paths that is of paramount importance in neurodegenerative diseases).

Acknowledgements

The studies described were supported with continuity since 1969 by CONICET, SECyTUNC, Fundación Antorchas and FONCyT, Argentina. Some studies along the years were done with support from the National Multiple Sclerosis Society, A.D. Williams Fund., MCV-VCU Fund, and NIH, U.S.A.

References

[1] G. Roberts, *Langmuir-Blodgett films*, Plenum Press, New York, 1990.
[2] E. Gorter & F. Grendel, *J. Exper. Med.* **41**, 439 (1925).
[3] G. Cevc & D. Marsh, *Phospholipid bilayers,* Wiley-Interscience, New York, 1987.
[4] J. N Israelachvili, S. Marcelja & R. G. Horn, *Quart. Rev. Biophys.* **13**, 121 (1980).
[5] M. C. Phillips, in J. F. Danielli, M. D. Rosenberg, & D. A. Cadenhead (Eds.), *Progress in surface and membrane science* (pp. 139–221), Academic Press, New York. 1972.
[6] B. Maggio, F. A.Cumar & R. Caputto, *Biochim. Biophys. Acta* **650**, 69 (1981).
[7] B. Maggio, *Progr. Biophys. Mol. Biol.* **62,**55 (1994).
[8] B. Maggio, D. C. Carrer, M. L. Fanani, R. G. Oliveira & C. M. Rosetti. *Curr. Op. Coll. & Interf.* Sci. **8**, 448 (2004).
[9] B. Maggio, M. L. Fanani, C. M. Rosetti & N. Wilke, *Biochim. Biophys. Acta* **1758**, 1922 (2006).
[10] B. Maggio, G.A. Borioli, M. Del Boca, L. De Tullio, M.L. Fanani, R.G. Oliveira, C.M. Rosetti & N. Wilke, *Cell Biochem. Biophys.* **50** 79 (2008).

[11] D. Marsh, *Biochim. Biophys . Acta* **1286**,183 (1996).
[12] S. Feng, *Langmuir* **15**, 998 (1999).
[13] M. C. Phillips, D. E.Graham & H. Hauser, *Nature* **254**, 154 (1975).
[14] E. Sackmann, In R. Lipowsky & E. Sackmann (Eds.), *Structure and dynamics of membranes: From cells to vesicles* (pp. 213–298), Elsevier, Amsterdam, 1995.
[15] M. L.Fanani & B. Maggio, *J. Lip. Res.* **41**, 1832 (2000).
[16] B. Maggio, J. Albert & R. K. Yu, *Biochim. Biophys. Acta* **945**, 145 (1988).
[17] M. A. Perillo, N. J. Scarsdale, R. K. Yu, & B. Maggio, *Proc. Nat .Acad. Sci. USA* **91**, 10019 (1994).
[18] G. Basañez, G. D. Fidelio, F. M. Goni, B. Maggio & A. Alonso *Biochemistry* **35**, 7506 (1996).
[19] A. Saez-Cirion, G. Basañez, G. D. Fidelio, F. M. Goñi, B. Maggio & A. Alonso, *Langmuir* **16**, 8958 (2000).
[20] B. Maggio & J.A. Lucy, *Biochem. J.* **155**,353 (1976).
[21] D. P. Siegel & R. M. Epand, *Biophys. J.* **73**, 3089 (1997).
[22] B. Maggio, M.G. Mestrallet F.A. Cumar & R. Caputto, *Biochim. Biophys. Res. Commm.* 1265 (1977)
[23] F. A. Cumar, B. Maggio & R. Caputto. *Biochim. Biophys. Acta* **597,** 174 (1980).
[24] B. Maggio & R. K. Yu, *Biochim. Biophys. Acta* **1112,** 105 (1992).
[25] B. Maggio, Q.F. Ahkong & J.A. Lucy, *Biochem. J.* **158,** 647 (1976).
[26] D. C. Carrer & B. Maggio, *Biochim. Biophys. Acta* **1514**, 87 (2001).
[27] C. G. Monferran, B. Maggio, G. A. Roth, F. A. Cumar & R. Caputto, *Biochim. Biophys. Acta* **553,** 417 (1979).
[28] N. Wilke & B .Maggio, *Biophys. Chem*. **122,** 36 (2006).
[29] X.-M. Li, J. M. Smaby, M. M. Momsen, H. L. Brockman & R.E. Brown, *Biophys. J.* **78,**1921 (2000)
[30] F. Dupuy, M.L. Fanani & B. Maggio, *Langmuir* **27,** 3783 (2011).
[31] B. Maggio, T. Ariga & R. K. Yu, *Biochemistry* **29**, 8729 (1990).
[32] B. Maggio, T. Ariga, R. O Calderón & R. K. Yu, *Chem. Phys. Lipids* **90,** 1 (1997).
[33] S. Härtel, M.L Fanani & B. Maggio, *Biophys. J.* **88,** 287 (2005).
[34] B. Maggio, *Chem. Phys. Lipids* **132**, 209 (2004).
[35] F. Dupuy & B. Maggio, *Chem. Phys. Lipids* **165,** 615 (2012).
[36] C.M. Rosetti, B. Maggio & R.G. Oliveira, *Biochim. Biophys. Acta* **1778,**1665 (2008).
[37] R.V. Vico, O.F. Silva, R.H. de Rossi & B. Maggio, *Langmuir,* **24**, 7867 (2008).
[38] R.V. Vico, R.H. de Rossi & B. Maggio, *Langmuir* **26,** 8407 (2010).
[39] D.C. Carrer, S. Schreier, M. Patrito & B. Maggio. *Biophys. J.* **90**, 2394 (2006).
[40] F.X. Contreras, A.V. Villar, A. Alonso, R N. Kolesnick, & F.M. Goni, *J. Biol. Chem*., **278,** 37169 (2003).
[41] M. L. Fanani, L.De Tullio, S. Hartel, J. Jara & B. Maggio, *Biophys. J.* **96**, 67 (2009).
[42] M. L. Fanani, S. Hartel, B. Maggio, L. De Tullio, J. Jara, F. Olmos & R. G. Oliveira, *Biochim. Biophys. Acta* **1798,** 1309 (2010).
[43] B. Maggio, *J. Lipid Res.* **40**, 930 (1999).
[44] N. Wilk & B. Maggio, *J. Phys. Chem. B* **11,** 12844 (2009).
[45] N. Wilke, S.A. Dassie, E.P.M. Leiva & B. Maggio, *Langmuir* **22,** 9664 (2006).
[46] R.G. Oliveira, R.O. Calderón & B. Maggio, *Biochim. Biophys. Acta.* **1370,** 127 (1998).
[47] R.O. Calderon, B. Maggio, T.J. Neuberger & G.H .DeVries, *J. Neurosci. Res.* **34,** 206 (1993).
[48] R.O. Calderon, B. Maggio, T.J. Neuberger & G.H. DeVries, *J. Neurosci. Res.* **40,** 349 (1995).

Manuscrito recibido el 6 de marzo de 2013.
Aceptado el 15 de abril de 2013.