Dynamic and Structural Properties of Sphingolipids as Driving Forces for the Formation of Membrane Domains

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1. Introduction

In 1972, S. J. Singer and G. L. Nicolson,[1] introducing the fluid mosaic model for the structure of cell membranes, reported on experimental data suggesting that some proteins aggregated in clusters under certain conditions. Then, investigations carried out in either artificial or cellular models using a variety of techniques suggested that organization in domains, whose properties are based on their peculiar lipid composition ("cholesterol-", "ceramide-", "glycolipid-", or "sphingolipid-"enriched membrane domains), might be a common feature of biological membranes. Currently, several authors accept as a working hypothesis that macro- and microdomains with molecular composition and physicochemical properties distinct from the surrounding membrane environment exist. However, most experimental evidence supporting this hypothesis is indirect (e.g., the study of detergent-insoluble membrane preparations) or in some aspects controversial (e.g., the size and lifetime of lipid-enriched membrane domains, the existence of heterogeneous membrane domain subpopulations), and the actual existence of lipid domains in cellular membranes is still strongly debated.[2] Indeed, the control of the localization and function of proteins that are components of signaling pathways regulated by the T-cell antigen receptor (TCR) is probably one of the biological events where the putative role of lipid-enriched membrane domains met the strongest consensus. In a Medline search, 215 papers dealing with this topic published from 1996 were found.[3] However, even this notion was recently challenged

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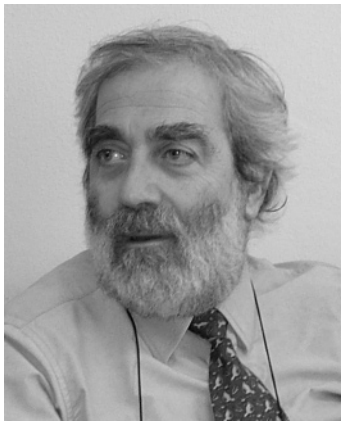

Sandro Sonnino, born in 1949, obtained at the University of Milan the M.S. Degree in Chemistry in 1973. He became Professor of Biochemistry at the School of Medicine, University of Milan, in 1990, where he is now. His scientific activity is all related to studies on sphingolipids. He has solved the primary structure of 11 glycosphingolipids and has studied the conformational, dynamic, geometrical, and aggregative properties of the main gangliosides from the nervous system, the role of gangliosides in the organization of sphingolipid-enriched membrane microdomains, the interaction processes between gangliosides and soluble or membrane proteins, and the metabolism and trafficking of gangliosides and their catabolites. He is Responsible for the "Glycobiology" group of the Italian Society of Biochemistry and Molecular Biology.

Alessandro Prinetti, born in 1965, obtained at the University of Milan the M.S. Degree in Pharmaceutical Chemistry in 1992 and the Ph.D. in Biochemistry in 1997. He became associate Professor in 2002 at the School of Medicine, University of Milan, where he is now. His main research topics were and are sphingolipid patterns and metabolism in neural cells during development and differentiation, the functional role of sphingoid molecules in the control of growth and differentiation of neural cells, the structure of sphingolipid-enriched membrane domains and their functional role in neural cell signaling, and the interaction between sphingolipids and Src family proteins.

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by experimental proofs arguing against the involvement of lipid membrane domains in TCR signaling.[4−6]

Thus, lipid-enriched membrane domains remain at least elusive, and even the forces that rule their basic organization, stability, and dynamics are only partly understood. Several topics related to this research area have been recently and extensively reviewed.[6−51] In this review, we do not intend to report general information on lipid membrane domains, but we intend to focus on some features of membrane sphingolipids that should be involved in the generation of lipid domains, aiming to show that extensive literature references demonstrate the primary role of the peculiar chemico-physical features of this class of lipids as a driving force in determining the properties of lipid membrane domains.

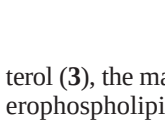
The mysterious aspect of lipid membrane domains is probably not surprising if we recall that sphingolipids, key components of lipid membrane domains together with cholesterol, associate their name with the enigma of the Sphinx.[52] Research on sphingolipids was introduced at the end of the nineteenth century by J. L. W. Thudichum, who

Guido Tettamanti, born in 1935, obtained the M.D. degree at the University of Pavia in 1960 and then the Ph.D. in Biochemistry in 1966. He became Professor of Medical Biochemistry at the Medical School of the University of Milan in 1975. He introduced in Italy research on gangliosides and organized the first group of scientists, chemists, physics, physicians, biologists, and biochemists all involved in the same project on the functional role of gangliosides. Organizer of many International Conferences in the field of gangliosides, he was Chairman of the Gordon Research Conference "Glycolipid and Sphingolipid Biology", Il Ciocco, Italy, 2000, and of the XVIII International Symposium on Glycoconjugates, Florence, Italy, 2005.

reported for the first time on the isolation of unknown compounds from human brain, whose structure had been enigmatic to him for a long time, before getting resolved. These compounds were then characterized to be cerebrosides, that is, monoglycosylceramides. Even if the newborn chemistry of sphingolipids attracted the attention of several investigators, progress in this field was extremely slow,[53] and the correct structure of sphingosine[54] was established only after half a century.

2. Membrane Lipids

The two highly heterogeneous groups of glycerophospholipids (**1**) and of sphingolipids (**2**) are, together with cholesterol (**3**), the main lipid components of cell membranes. Glycerophospholipids are by far the main lipids of eukaryotic cell membranes. Sphingolipids are minor cell components, mainly residing in the external layer of the plasma membrane[55] with the hydrophilic headgroup protruding toward the extracellular environment. Many reports indicated multiple functional roles for membrane sphingolipids, including the modulation of the properties of important membrane enzymes.[56−72]



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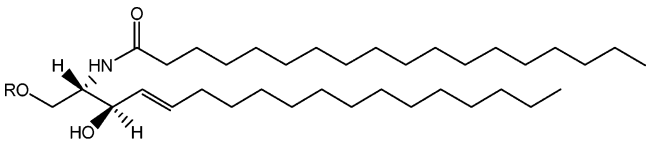

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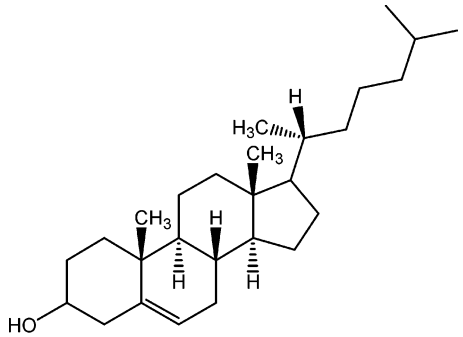
Table 1. Structures of the Main Neutral Glycosphingolipids in Vertebrates[a]

series	abbreviation	structure
	GalCer	β-Gal-(1−1)-Cer
	GlcCer	β-Glc-(1−1)-Cer
	LacCer	β-Gal-(1−4)-β-Glc-(1−1)-Cer
ganglio-3	Gg_3Cer	β-GalNAc-(1−4)-β-Gal-(1−4)-β-Glc-(1−1)-Cer
ganglio-4	Gg_4Cer	β-Gal-(1−3)-β-GalNAc-(1−4)-β-Gal-(1−4)-β-Glc-(1−1)-Cer
ganglio-5	Gg_5Cer	β-GalNAc-(1−4)-β-Gal-(1−3)-β-GalNAc-(1−4)-β-Gal-(1−4)-β-Glc-(1−1)-Cer
globo-3	Gb_3Cer	α-Gal-(1−4)-β-Gal-(1−4)-β-Glc-(1−1)-Cer
globo-4	Gb_4Cer	β-GalNAc-(1−3)-α-Gal-(1−4)-β-Gal-(1−4)-β-Glc-(1−1)-Cer
globo-5	Gb_5Cer	β-Gal-(1−3)-β-GalNAc-(1−3)-α-Gal-(1−4)-β-Gal-(1−4)-β-Glc-(1−1)-Cer
isoglobo-3	iGb_3Cer	α-Gal-(1−3)-β-Gal-(1−4)-β-Glc-(1−1)-Cer
lacto (paraglobo)	Lc_4Cer	β-Gal-(1−3)-β-GlcNAc-(1−3)-β-Gal-(1−4)-β-Glc-(1−1)-Cer
neolacto-4	nLc_4Cer	β-Gal-(1−4)-β-GlcNAc-(1−3)-β-Gal-(1−4)-β-Glc-(1−1)-Cer
neolacto-6	nLc_6Cer	β-Gal-(1−4)-β-GlcNAc-(1−3)-β-Gal-(1−4)-β-GlcNAc-(1−3)-β-Gal-(1−4)-β-Glc-(1−1)-Cer

[a] Nomenclature is according to Svennerholm and the IUPAC−IUB indications.[254−255]



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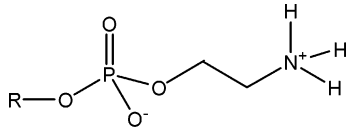
The lipid moiety of sphingolipids, **2**, is named ceramide. Ceramide is constituted by a long chain amino alcohol,[73] 2-amino-1,3-dihydroxy-octadec-4-ene, whose trivial name is sphingosine, connected to a fatty acid by an amide linkage. Of the four possible configurations of sphingosine, only the 2*S*,3*R* is present in nature.[54,74] The same trivial name is used also for structures with shorter and longer alkyl chains and for structures lacking the double bond (whose trivial name should be more properly sphinganine).

The hydrophilic headgroup R of sphingolipids is phosphocholine in the case of sphingomyelin, **4**, or an oligosac-



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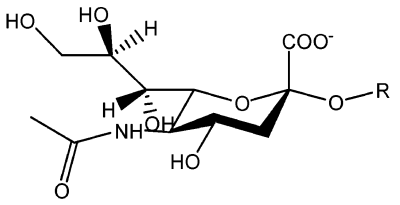
charide chain. When the oligosaccharide chain carries residues of sialic acid[75] (or neuraminic acid, the trivial names used for the derivatives of 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid), the glycosphingolipid is named ganglioside. The three most represented structures of sialic acid are the 5-*N*-acetyl- (**5**), the 5-*N*-acetyl-9-*O*-acetyl- (**6**), and the 5-*N*-glycolyl- (**7**) derivatives, but the latter is absent in normal human tissues.[76,77] In humans, about 10% of the total ganglioside-bound sialic acid is the 9-*O*-acetyl-*N*-acetylneuraminic acid,[77] and polysialogangliosides that contains this structure have been characterized in mice brains.[78,79] Ester linkages in polysialyl chains containing compounds such as **8** have been also characterized.[80]



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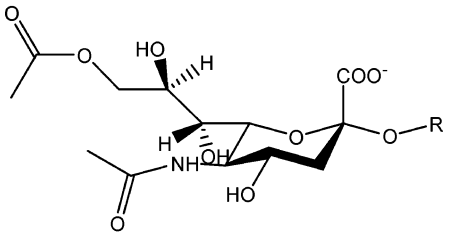

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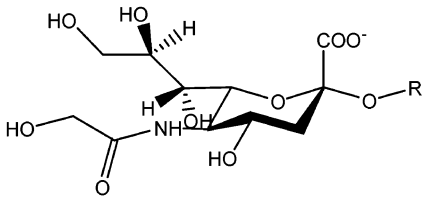

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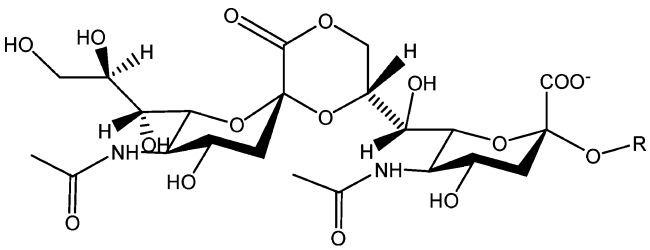
Tables 1, 2, and 3 show the main oligosaccharide structures grouped for neutral glycolipids, gangliosides, and sulfoglycolipids, respectively.



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3. Membrane Lipid Domains

The interest in membrane lipid domains, that is, in zones of the membrane with a peculiar lipid composition, different

Table 2. Structures of the Main Gangliosides of the Nervous System[a]

GM4	Neu5AcGalCer
GM3	II^3Neu5AcLacCer
GD3	II^3(Neu5Ac)$_2$LacCer
O-acetyl-GD3	II^3[Neu5,9Ac$_2$-(2−8)-Neu5Ac]LacCer
GM2	II^3Neu5AcGg$_3$Cer
GD2	II^3(Neu5Ac)$_2$Gg$_3$Cer
GM1	II^3Neu5AcGg$_4$Cer
GM1b	IV^3Neu5AcGg$_4$Cer
Fuc-GM1	$IV^2\alpha$FucII^3Neu5AcGg$_4$Cer
GalNAc-GM1	II^3Neu5AcGg$_5$Cer
3′-LM1	IV^3nLc$_4$Cer
GD1a	IV^3Neu5AcII^3Neu5AcGg$_4$Cer
GD1α	IV^3Neu5AcIII^6Neu5AcGg$_4$Cer
GalNAc-GD1a	IV^3Neu5AcII^3Neu5AcGg$_5$Cer
GD1b	II^3(Neu5Ac)$_2$Gg$_4$Cer
GD1b-lactone	II^3[Neu5Ac-(2−8,1−9)-Neu5Ac]Gg$_4$Cer
Fuc-GD1b	$IV^2\alpha$FucII^3Neu5Ac$_2$Gg$_4$Cer
GT1a	IV^3(Neu5Ac)$_2II^3$Neu5AcGg$_4$Cer
GT1b	IV^3Neu5AcII^3(Neu5Ac)$_2$Gg$_4$Cer
O-acetyl-GT1b	IV^3Neu5AcII^3[Neu5,9Ac$_2$-(2−8)-Neu5Ac]Gg$_4$Cer
GT1c	II^3(Neu5Ac)$_3$Gg$_4$Cer
Chol-1α-a	IV^3Neu5AcIII^6Neu5AcII^3Neu5AcGg$_4$Cer
Chol-1β	III^6Neu5AcII^3(Neu5Ac)$_2$Gg$_4$Cer
GT1α	IV^3Neu5AcIII^6(Neu5Ac)$_2$Gg$_4$Cer
GQ1b	IV^3(Neu5Ac)$_2II^3$(Neu5Ac)$_2$Gg$_4$Cer
GQ1c	IV^3Neu5AcII^3(Neu5Ac)$_3$Gg$_4$Cer
GQ1α	IV^3(Neu5Ac)$_2III^6$(Neu5Ac)$_2$Gg$_4$Cer
Chol-1α-b	IV^3Neu5AcIII^6Neu5AcII^3(Neu5Ac)$_2$Gg$_4$Cer
GP1c	IV^3(Neu5Ac)$_2II^3$(Neu5Ac)$_3$Gg$_4$Cer

[a] Sialic acid in gangliosides is only in configuration α. Chetosidic linkage to sialic acid is always 2 → 8. The roman numeral identifies the sugar unit, starting from ceramide, to which sialic acid, the sialosyl chain, is linked; the superscript Arabic number identifies its linkage position.

from that of the majority of bilayer, became very strong during the last 15 years, when many proteins assigned to cell signaling were found to be preferentially associated with an environment of lipids highly enriched in sphingolipids and cholesterol. A possible role for lipid domains in the transport of glycosylphosphatidylinositol (GPI)-anchored proteins from the Golgi apparatus to the apical plasma membrane of polarized cells was also suggested; hence, the use of the term "lipid rafts" to define these domains.[81]

Biochemical studies on cell membrane lipid domain composition, organization, and biological role were carried out mainly starting from 1992, when a method capable of separating them from total cell membranes became available.[82] However, massive biophysical work on artificial membranes was carried out from the 1970s to understand the basis of the organization of amphiphilic compounds within biological membranes, leading to establishment of the existence of segregation phenomena.

Today, different terms are used throughout the literature to define lipid membrane domains. Every one of them implies a form of segregation of certain components within the cell membrane. Some define specific domains, as in the case of "caveolae", membrane invaginations containing the protein caveolin,[83] or "lipid rafts", membrane domains that sort or transport proteins inside cells. Others define membrane portions on the basis of chemico-physical or compositional features, such as "DIM" (detergent-insoluble material),[84] "DISAM" (detergent-insoluble substrate attachment matrix),[84] "DIG" (detergent-insoluble glycolipid-enriched material),[85] "DRM" (detergent-resistant membranes),[86] "SEMF" (sphingolipid-enriched membrane fraction),[87] "GSD" (glyco signaling domains),[88] etc. To these authors, DRM is a very reasonable definition, based on the operational way commonly used to obtain biochemical preparation enriched in lipid membrane domains, which is based on their relative insolubility in nonionic detergents under specific experimental conditions.

3.1. Artificial Membranes

The earliest evidence supporting the existence of lipid domains, conceived as areas in the membrane different in lipid composition from other areas in the membrane, was obtained studying artificial membrane models. The main experimental membrane model that has been exploited for these investigations is represented by phospholipid bilayers, usually in the form of glycerophospholipid vesicles or liposomes containing glycosphingolipids, sphingomyelin, ceramide, or cholesterol. Valuable pieces of information were also obtained from different artificial membrane models, including sphingolipid micelles, phospholipid bilayers on solid support, and lipid monolayers on an air/water interface or on solid support. Very heterogeneous analytical techniques are suitable for each of these models, contributing to the difficulty sometimes encountered in comparing and generalizing the results obtained by specific experimental approaches.

Lipid bilayers usually exist in an ordered gel phase at low temperatures. Above a melting temperature (T_m) characteristic of each molecular species of lipid, the bilayer is present in the so-called liquid-crystalline (lc) or liquid-disordered (ld) phase, in which the lipid acyl chains are disordered and characterized by high fluidity. Membrane lipids can also exist in a third physical phase, the liquid-ordered (lo) phase. Acyl chains of lipids in the lo phase have properties that are intermediate between those of the gel and ld phases. They are extended and ordered, as in the gel phase but have high lateral mobility in the bilayer, as in the ld phase.[89] The coexistence of lipids in different phases within the same model membrane was probably the first evidence leading to the concept of lipid domains. Incomplete miscibility within a single phase, leading to lateral phase separation of complex lipids in phospholipid bilayers has been extensively studied by X-ray diffraction,[90,91] differential scanning calorimetry (DSC),[90,93−100] freeze-etch electron microscopy (EM),[91,101−105] spontaneous interbilayer transfer rate measurements,[106] conductance measurements,[107] electron spin resonance (ESR),[91,108] fluorescence microscopy,[109] fluorescence correlation spectroscopy,[110] fluorescence resonance energy transfer (FRET)[111] and fluorescence recovery after photobleaching (FRAP),[112−115] and nuclear magnetic resonance (NMR).[116,117]

Lateral phase separation is a feature not restricted to sphingolipids. Indeed phase separation can be observed in binary mixtures of dialkyl lecithins differing in chain length or saturation[101,118,119] and in ternary mixtures of palmitoyloleyl phosphatidylcholine (PC), dioleyl PC, and cholesterol.[109] However, in the case of glycosphingolipids, their unique molecular structure and conformational properties suggested a strong tendency to form segregated compositional domains in phospholipid bilayers. Starting from the early 1980s, this was clearly shown for a number of neutral glycosphingolipid molecular species,[120] including synthetic galactosyl(*N*-palmitoyl)sphingosine[91] and bovine brain galactosylceramide,[121] synthetic glucosyl(*N*-palmitoyl)sphingosine[122] and Gaucher spleen glucosylceramide,[92,106,107,123] globoside,[91] and asialo GM1.[101−103] Similar results were obtained for charged glycosphingolipids such as sulfati-

Table 3. Sructure of the Main Sulfatides

SM4s	$^{-}O_3S$-3-β-Gal-(1−1)-Cer
SM4s-6	$^{-}O_3S$-6-β-Gal-(1−1)-Cer
SM4s-Glc	$^{-}O_3S$-3-β-Glc-(1−1)-Cer
SM3	$^{-}O_3S$-3-β-Gal-(1−4)-β-Glc-(1−1)-Cer
SM2a	β-GalNAc-(1−4)-[$^{-}O_3S$-3-]-β-Gal-(1−4)-β-Glc-(1−1)-Cer
SM2b	$^{-}O_3S$-3-β-GalNAc-(1−4)-β-Gal-(1−4)-β-Glc-(1−1)-Cer
SB1a	$^{-}O_3S$-3-β-Gal-(1−3)-β-GalNAc-(1−4)-[$^{-}O_3S$-3-]-β-Gal-(1−4)-β-Glc-(1−1)-Cer
$SMGb_4$	$^{-}O_3S$-3-β-GalNAc-(1−3)-α-Gal-(1−4)-β-Gal-(1−4)-β-Glc-(1−1)-Cer
$SMGb_5$	$^{-}O_3S$-3-β-Gal-(1−3)-β-GalNAc-(1−3)-α-Gal-(1−4)-β-Gal-(1−4)-β-Glc-(1−1)-Cer
	$^{-}O_3S$-3-β-Gal-(1−3)-β-GalNAc-(1−4)-[α-Neu5Gc-(2−3)-]-β-Gal-(1−4)-β-Glc-(1−1)-Cer
$SMUnLc_4Cer$	$^{-}O_3S$-3-β-GlcU-(1−3)-β-Gal-(1−4)-β-GlcNAc-(1−3)-β-Gal-(1−4)-β-Glc-(1−1)-Cer
$SMUnLc_6Cer$	$^{-}O_3S$-3-β-GlcU-(1−3)-β-Gal-(1−4)-β-GlcNAc-(1−3)-β-Gal-(1−4)-β-GlcNAc-(1−3)-β-Gal-(1−4)-β-Glc-(1−1)-Cer

des.[93,108] Much more controversial appeared the situation for gangliosides, which are unique among glycosphingolipids for their aggregative properties in aqueous solutions.[124] Pioneering work using spin-label probes[125,126] showed that gangliosides, even at low concentration, reduced fluidity and hydrocarbon chain mobility in phosphatidylcholine bilayers, due to lateral cooperative interactions between ganglioside molecules. This behavior has been interpreted in the sense of a tendency of gangliosides to form "clusters" in fluid lipid bilayers, that is, lipid domains, and attracted very much attention for its possible biological relevance in determining ganglioside functions.[126,127] Ca^{2+} addition caused a further decrease of membrane fluidity, suggesting that interactions involving ganglioside headgroups could be the driving force for ganglioside phase separation.[126] These results were apparently challenged by other studies indicating that at low concentration of gangliosides these components are dispersed in phosphatidylcholine bilayers.[128−132] EM analysis coupled with labeling with cholera toxin or specific IgG and Fab fragments showed that GM1 ganglioside and the neutral Forssman glycosphingolipid are randomly distributed in phospholipid bilayers, whereas the neutral glycosphingolipid asialo-GM1 is present in microdomains of about 16 molecules.[103,104,133,134] When similar studies were performed using multilamellar liposomes composed of 1:1 dielaidoylphosphatidylcholine/dipalmitoylphosphatidylcholine, which exhibit laterally separated fluid- and gel-phase regions (an artificial model more closely simulating the situation in biological membranes, where the membrane contains more than one species of phospholipid), incorporating minor amounts of different glycosphingolipids, it has been shown that ganglioside GM1 and its neutral derivative asialo-GM1 distribute preferentially into gel-phase regions, whereas Forssman glycolipid distributes evenly in the two phases.[101] This observation suggested that the contrast in behavior between these lipids could be ascribed to the differences in the hydrophobic moieties (i.e., in the acyl chain composition). These issues could be properly addressed thanks to the availability of semisynthetic ganglioside species differing in ceramide acyl chain length, degree of saturation, and nature of the sphingoid base.[135] The preparation and use of liposomes for the study of sphingolipid segregation in membrane model systems[136,137] unambiguously showed the following: (1) Ganglioside phase separation occurs in two-component, two-phase, as well as in one component, phosphatidylcholine bilayers.[94−97,105,138,139] Interestingly, GM1-enriched domains are also present in sphingomyelin bilayers.[99] (2) For a given oligosaccharide composition, the extent of ganglioside lateral phase separation depends on the length and unsaturation difference between the ganglioside long-chain base and phosphatidylcholine acyl chains.[94−96] (3) For GM1 species with homogeneous sphingoid base composition, a decrease in the acyl chain length or an increase in its unsaturation induced increased ganglioside distribution in the liquid phase of the bilayer.[105] (4) For a given lipid moiety composition, the extent of ganglioside phase separation is dependent upon the number of sugars in the oligosaccharide headgroup.[95,96] (5) The addition of Ca^{2+} promotes phase separation.[126] This is not due to a Ca^{2+}-induced bridging between ganglioside headgroups but rather to a passive ganglioside exclusion from phosphatidylcholine-rich regions of the bilayer, which are perturbed by Ca^{2+}.[94,96]

At the end of the 1980s, the discovery that ceramide could represent an important cell mediator in a rapidly growing number of different physiological, pharmacological, and pathological situations shifted at least in part the interest of researchers from glycosphingolipids toward simpler sphingoid molecules and sphingomyelin as a possible source for those molecules. Not surprisingly, the ability to undergo lateral phase separation in phospholipid vesicles proved to be not restricted to glycosphingolipids. Sphingomyelin undergoes lateral phase separation in dimyristoylphosphatidylcholine bilayers, depending on the degree of sphingomyelin chain mismatch:[140] the segregation tendency is very poor for *N*-palmitoyl-sphingomyelin[112,140] but is clear for sphingomyelin with a C24 acyl chain.[140] Lateral separation of ceramide-rich phases in phospholipid bilayers with as little as <5 mol % ceramide was observed for natural ceramides from different sources and for *N*-palmitoyl-ceramide.[100,112,141,142] When the behavior of *N*-palmitoyl-sphingomyelin and *N*-palmitoyl-ceramide in 1-palmitoyl-2-oleoyl-phosphatidylcholine liposomes was compared, ceramide showed a much more pronounced tendency to undergo lateral phase separation, suggesting that the enzymatic conversion of sphingomyelin to ceramide at the cell surface could be responsible for a membrane reorganization with formation of ceramide-rich membrane domains.[100,112]

The success of these experimental approaches stimulated their use to address more complex situations, such as the behavior of phospholipid bilayers in the presence of cholesterol or sphingomyelin, with or without gangliosides. These models seem closer to a natural cell membrane and allow the study of the reciprocal interactions of lipids that are normally colocalized in biochemical membrane preparations putatively containing natural lipid membrane domains (see section 3.2). The study of miscibility phase diagrams of giant vesicles containing sphingomyelin (SM), unsaturated PC (either palmitoyloleylPC or dioleylPC), and cholesterol suggested the coexistence of solid, liquid-ordered, and liquid-disordered phases in ternary mixtures both in the presence and in the absence of SM.[109] Cholesterol by itself forms a cholesterol-rich liquid-ordered phase and a phosphatidylcholine-rich liquid-disordered phase when present in the molar range of 5−25% in phosphatidylcholine bilayers[143]

and a cholesterol-enriched phase in palmitoylsphingomyelin vesicles.[99] Similar cholesterol-rich ordered liquid phases were formed in phospholipid bilayers in the presence of sphingomyelin, which mixes more ideally with cholesterol than a phosphatidylcholine with the same acyl chain.[98,144] Recent studies with fluorescent lipid derivatives highlighted the importance of the hydrophobic mismatch of the constituents for the colocalization of cholesterol and sphingomyelin in a phospholipid bilayer,[113] arguing against the supposed existence of a specific interaction between cholesterol and sphingomyelin (see, for example, ref 145, and further discussion below). When ternary sphingomyelin/GM1/cholesterol vesicles were analyzed by DSC, the formation of separate GM1-enriched and cholesterol-enriched domains was shown.[99]

More recently, complex liposomes composed of glycerophospholipids, sphingomyelin, and cholesterol were used to study the dynamics of changes in lateral phase separation occurring upon modification of the sphingomyelin/ceramide content or ratio by addition of ceramide to preformed liposomes or enzymatic hydrolysis of sphingomyelin. The results of these works showed that increased amounts of ceramide in liposomes induce dramatic changes in the bilayer organization, possibly as a consequence of the lateral phase separation of ceramide-rich domains.[114,146,147]

As mentioned above, gangliosides have unique aggregative properties, and in diluted aqueous solution, they generally form micelles of large molecular weight.[124] This feature allowed attainment of further information about the lateral segregation of gangliosides using mixed micellar systems, which can be conveniently studied by laser light scattering. In mixed micelles of two gangliosides, GM2 and GT1b, characterized by similar hydrophobic moiety composition and very different hydrophilic headgroups, GT1b and GM2 monomers are not randomly distributed in the micelle.[148] The segregation of one ganglioside with respect to the other in this artificial system is a spontaneous process explained on the basis of the different geometrical properties of ganglioside headgroups. A similar segregation as well due to the geometrical differences between these two gangliosides was shown in mixed micelles of GD1b and GD1b-lactone.[149]

Monomolecular lipid films ("monolayer membranes") at the air/water interface have been used in particular to study the lateral domain formation ability of cholesterol in phospholipid membranes and the role of sphingomyelin−cholesterol interactions in the formation of segregated compositional lipid domains. The properties of lipid monolayers can be studied by measuring physico-chemical parameters such as surface pressure and molecular areas[150−153] or by fluorescence microscopy.[154−157] Several studies indicated that cholesterol preferentially interacts with sphingomyelin in mixed monolayers.[150−156,158,159] This interaction occurs independently from the acyl chain length of sphingomyelin[152,158] but was lower in the presence of a cis-unsaturation.[99,101,104] The apparent strong interaction between cholesterol and sphingomyelin would be the main one responsible for the formation of liquid-condensed lateral cholesterol- and sphingomyelin-rich domains, which can very efficiently incorporate GM1 at low concentration[156,160] and are resistant to Triton X-100 solubilization[152,153] as are DRM in natural membranes. However, as mentioned before, a recent paper challenged this concept,[113] showing that in phospholipid bilayers there is no evidence of such a specific interaction between cholesterol and sphingomyelin.

An interesting recent evolution of membrane models for the study of lipid lateral segregation is represented by the use of solid-supported lipid mono- or bilayers. The properties of putative DRMs reconstituted on Langmuir monolayers transferred from the air−water interface to a silanized glass support have been studied using fluorescence microscopy.[115] Lipid monolayers prepared from mixtures of synthetic lipids as well as by natural cell lipid extracts undergo spontaneous phase separation with the formation of a cholesterol- and GM1-enriched liquid-ordered phase. Cholesterol density can be manipulated in this membrane model by treatment with cyclodextrins, allowing the conclusion that cholesterol density is a critical parameter for the existence of lateral lipid domains. This model is very appealing because its composition can be chosen to approximate very well the cell lipid composition, it can be manipulated quite conveniently, and it is possible to complicate it by the addition of proteins whose partition into the lateral lipid domains can be easily studied. However, possibly the most promising application of solid-supported lipid mono-[161−163] and bilayers[164−169] is represented by their coupling with atom force microscopy (AFM).[161−170] AFM on such artificial membranes indeed largely confirmed the results obtained using different membrane models. However, the advantages of this technique are represented by the possibility to investigate the properties of very complex lipid mixtures, also including proteins.[167] The lipid composition of the artificial membrane can be easily manipulated and analyzed in real time[163] with a power of resolution in the submicrometer range.[161,164] Thus, these model systems seem to be adequate to study the up-to-now highly controversial size and time scales of lipid domains, an issue that probably represents the next challenge in the study of lipid domains.

3.2. Natural Membranes

The existence of lipid membrane domains in natural cell membranes was suggested by the observation that glycosphingolipids at the cell surface form clusters, which have been visualized by immuno-electron microscopy using anti-glycosphingolipid antibodies.[171] Glycosphingolipid clustering in cell membranes was shown for globoside in human erythrocytes,[172] polysialogangliosides in fish brain neurons,[173] GM3 ganglioside in peripheral human lymphocytes, and Molt-4 lymphoid cells.[174] Several approaches, relying on more advanced technologies, are now available allowing the detection and the study of membrane lipid domains in intact cell membranes.[22,23]

These techniques are very heterogeneous and include single-particle tracking or single-fluorophore tracking microscopy,[175−177] fluorescence recovery after photobleaching,[178] fluorescence resonance energy transfer,[179] and atom force microscopy,[180] and data obtained with different approaches are sometime conflicting. As an example, there is no agreement on their average size, which ranges from 26 nm to about 2 μm.[177−181] In addition, from these data no information on what composes the lipid domains can be inferred.

Compositional information for membrane lipid domains is largely obtained by means of the procedure published by Brown and Rose,[82] based on the insolubility in aqueous nonionic detergents of those cell lipids (including cholesterol, sphingolipids, and saturated PC) that in membrane models tend to segregate into a liquid-ordered phase. All the complex amphiphilic compounds that are components of cell mem-

Table 4. Composition of the Membrane Fraction Containing Lipid Domains (LD) and of the Cell Homogenate from Undifferentiated, Differentiated, and Aged Rat Cerebellar Granule Cells in Culture (2nd, 8th, and 17th Day in Culture (DIC), Respectively)[a]

	lipid domain fraction (nmol/10^6 cells) DIC			cell homogenate (nmol/10^6 cells) DIC			lipid domain fraction (% on all components) DIC			cell homogenate (% on all components) DIC		
	2nd	8th	17th	2nd	8th	17th	2nd	8th	17th	2nd	8th	17th
proteins	0.00	0.02	0.03	0.44	1.25	1.43	0.1	0.3	0.3	2.8	2.9	2.5
ceramide	0.02	0.11	0.17	0.04	0.22	0.34	1.0	1.5	1.5	0.2	0.5	0.6
sphingomyelin	0.13	0.67	0.63	0.26	1.00	1.05	8.3	9.4	5.7	1.6	2.3	1.8
gangliosides	0.04	0.47	0.43	0.07	0.79	1.02	2.2	6.6	3.9	0.5	1.8	1.8
GM3	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
GM1	0.001	0.04	0.02	0.003	0.06	0.05	0.05	0.56	0.18	0.02	0.14	0.09
GD3	0.004	0.02	0.02	0.011	0.04	0.04	0.22	0.28	0.18	0.08	0.09	0.07
GD1a	0.013	0.11	0.12	0.020	0.21	0.26	0.71	1.54	1.09	0.14	0.48	0.46
GD1b	0.005	0.05	0.04	0.009	0.09	0.10	0.27	0.72	0.36	0.06	0.20	0.18
O-Ac-GT1b	0.002	0.06	0.03	0.003	0.08	0.09	0.11	0.84	0.27	0.02	0.18	0.16
GT1b	0.007	0.17	0.12	0.025	0.26	0.32	0.38	2.39	1.09	0.18	0.59	0.56
O-Ac-GQ1b	*b*	*b*	*b*	0.001	0.01	0.01	*b*	*b*	*b*	0.01	0.03	0.02
GQ1b	0.001	0.02	0.01	0.002	0.02	0.03	0.04	0.28	0.09	0.01	0.06	0.05
cholesterol	0.70	1.91	1.71	2.77	4.80	5.02	44.2	26.8	15.5	17.4	11.2	8.7
glycerolipids	0.77	4.07	9.03	12.05	31.73	46.39	44.2	55.4	73.0	77.4	76.5	80.4
PE	0.02	0.35	2.12	2.47	6.64	12.30	2.6	8.6	23.5	20.5	20.9	26.5
PPE	0.02	0.05	0.50	0.45	4.13	3.20	2.6	1.2	5.5	3.7	13.2	6.9
PC	0.65	3.41	6.03	6.42	16.41	24.59	84.4	84.2	66.8	53.3	51.7	53.0
PPC	0.03	0.01	0.32	1.44	0.35	1.48	3.9	0.2	3.5	11.9	1.1	3.2
PS	0.04	0.18	*b*	0.75	2.59	3.28	5.2	4.4	*b*	6.2	8.2	7.1
PI	0.01	0.05	0.14	0.52	1.22	1.54	1.3	1.2	1.5	4.3	3.8	3.3
PPI	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
PIP	*b*	0.01	*b*	*b*	0.15	*b*	*b*	0.2	*b*	*b*	0.5	*b*
PIP2	*b*	0.01	*b*	*b*	0.24	*b*	*b*	0.2	*b*	*b*	0.8	*b*

[a] Reprinted in modified form with permission from refs 87 and 214. Copyright 2000 and 2001 American Society of Biochemistry and Molecular Biology. [b] Traces or under detection.

branes and several intrinsic membrane proteins can be solubilized by detergents, due to the formation of mixed aggregates. But, under certain experimental conditions, where low temperature, detergent concentration, and detergent-to-cell ratio seem the most critical parameters, a part of the cell membrane remains insoluble in the nonionic detergent Triton X-100. Other detergents were used and claimed to be not capable of solubilizing membrane lipid domains.[182−186] After detergent treatment, the detergent-insoluble membrane fraction can be separated from the rest of the cell thanks to its relative light density (buoyancy),[187−200,245] using continuous or discontinuous sucrose density gradients. The low density of the detergent-insoluble material is due to the high lipid-to-protein ratio in the fraction. Low-density, detergent-insoluble fractions were isolated from a wide variety of cultured cells, including almost all normal and pathological mammalian cell types investigated so far, as well as yeasts[201] and protozoans.[202] In a second time, reports on the isolation on this fraction from tissues appeared.[204−209]

Membrane lipid domains contain all the cell complex lipids but are highly enriched in sphingolipids and cholesterol. Nevertheless, glycerophospholipids remain the bulk components, being enriched in phosphatidylcholine (PC) and PC being highly enriched in the dipalmitoyl species. These results were confirmed using other procedures where the detergent is substituted by high pH, hypertonic sodium carbonate,[210] or mechanical treatments (sonication under carefully controlled conditions),[211] followed by density gradient centrifugation to recover the light fraction. The general agreement on the sphingolipid and cholesterol enrichment in the low-density membrane fraction obtained with the above procedures, that is, under different experimental conditions, would prove that the isolated lipid membrane domains are not the result of a random rearrangement of cell components induced by the treatment but are a good mirror of an organization naturally occurring on the cell membranes. Of course, any change in the experimental conditions can yield quantitative changes of each component.

In Table 4, we report the composition of DRM prepared from neurons, rat cerebellar granule cells, at different stages of differentiation in culture. This, to the best of our knowledge, is the most detailed quantitative and qualitative composition of DRM.

Membrane lipid domains contain a small amount of proteins, in general, no more than 0.5−2.5% of total cell proteins. However, proteins that are involved in the process of transduction of the information throughout the cell plasma membrane (including receptor and nonreceptor protein tyrosine kinases, G protein coupled receptors, GTP binding proteins) result frequently enriched in the lipid domains.

Scientists are making every effort to isolate different membrane lipid domains from the DRM fraction. Immunoisolation procedures allowed fractionation of the DRM fraction into subpopulations with strikingly different structure and functions. Two membrane subfractions were separated from DRM isolated from B16 melanoma cells by anti-GM3 ganglioside monoclonal antibody DH2 and by anti-caveolin antibody.[212] The anti-GM3 subfraction was enriched in GM3 and contained sphingomyelin, cholesterol, and proteins c-Src and Rho A but not caveolin, while the anti-caveolin subfraction contained caveolin, glucosylceramide, sphingomyelin, and a large amount of cholesterol, but not GM3, c-Src, or Rho A.[213] The GM3-enriched subfraction, but not the caveolar subfraction, was involved in cell adhesion-dependent signal transduction in these cells. Two subpopulations, containing two functionally different neuronal GPI-anchored proteins, Thy-1 and PrP,[206] were separated from the detergent-resistant fraction isolated from mouse brain.

Immunoisolation approaches based on the use of different antibodies against protein or lipid components of the lipid

Table 5. Transition Temperature, T_t (°C), Hydrodynamic Radius, R_h (Å), Axial Ratio, R_a/R_b, and Molecular Mass, M (kDa), of the Aggregates and Surface Area, a_0 (Å^2), of the Monomer in Aggregate[a]

	T_t							
	1	2	3		R_h	R_a/R_b	M	a_0
GlcCer from spleen	83.7							
LacCer from bovine adrenal medulla	74.4							
Gg_3Cer by hydrolysis of bovine brain GM2	60.8							
Gg_4Cer by hydrolysis of bovine brain GM1	54.0							
GM4 from bovine brain				vesicle	~300		18270	~80
GM3 from bovine adrenal medulla	35.3							
GM3 from bovine brain				vesicle	~250		16700	~80
GM2 from bovine brain	29.3			micelle	66.0	3.1	740	92.0
GM1 from bovine brain	19.3	19.7		micelle	58.7	2.3	470	95.4
GM1(d18:1,18:0) previously warmed at 60 °C		11.7		micelle	52.8			
GM1(d18:1,18:0) previously warmed at 40 °C		15.0		micelle	56.2			
GM1(d18:1,18:0) previously warmed at 25 °C		17.6		micelle	58.7			
GM1(d20:1,18:0) previously warmed at 60 °C		23.2		micelle				
FucGM1 from pig brain		13.2		micelle	61.0	2.1	394	97.8
FucGM1(d18:1,18:0) previously warmed at 60 °C		10.0		micelle				
FucGM1(d20:1,18:0) previously warmed at 60 °C		18.3		micelle				
GD1a from bovine brain	15.2	16.0		micelle	58.0	2.0	418	98.1
GD1a(d18:1,18:0) previously warmed at 60 °C		10.1		micelle				
GD1a(d20:1,18:0) previously warmed at 60 °C		19.2		micelle				
GalNAcGD1a from bovine brain				micelle	60.0		509	97.0
GD1b from bovine brain				micelle	52.0	1.8	311	100.8
GD1b-lactone synthesis from bovine brain GD1b				micelle	57.0	2.1	424	97.6
GT1b from bovine brain	7.3			micelle	53.2	1.8	378	100.8
SM from several sources			35.7 ± 5.8	vesicle				

[a] Natural compounds have a heterogeneous ceramide moiety.[216−219] Reprinted in modified form with permission from ref 124. Copyright 1994 Elsevier.

membrane domain fraction might thus become the method of choice for the separation of lipid membrane domains, possibly overcoming some of the negative aspects of the classical density gradient flotation method after detergent lysis and allowing attainment of novel information about the properties of functionally and structurally different subpopulations of lipid membrane domains. The use of antibodies could however be responsible for artifactual clustering induced by antibody bridging or cross-linking; hence caution should be exerted also in this case.

4. The Driving Forces Leading to Lipid Segregation

Membrane lipid domains are highly enriched in sphingomyelin, glycosphingolipids, cholesterol, and dipalmitoylphosphatidylcholine, one of the molecular species of phosphatidylcholine.[214] On the basis of the number of lipid molecule components of the detergent-resistant membrane fraction, they roughly should cover 10−20% of the cell surface.[214] On the other hand, lipid domains contain only 0.5−2.5% of the total cell proteins. Thus, even if specific interactions between proteins and lipids likely play a relevant role in the membrane domain organization, we can predict that lipid chemical and physico-chemical properties are the driving forces governing the membrane domain existence and organization. In the following paragraphs, we discuss transition temperature of membrane lipids, the hydrogen bond network at the lipid−water interface, the geometrical properties of hydrophilic headgroups of membrane lipids, the side-by-side oligosaccharide interactions, and the carbohydrate−water interactions, as driving forces to segregation phenomena.

4.1. The Lipid Transition Temperature

Membrane complex lipids are highly heterogeneous in their lipid moieties. There are a large number of species containing unsaturated alkyl chains. This it is believed to be an essential requirement to organize a dynamic membrane system that allows protein conformational changes and lipid organization changes. But, lipids with less fluid saturated chains are also membrane components. High transition temperature of the lipid chain of complex lipids should be one of the most important properties of the components belonging to the membrane lipid domains.[171] In a membrane, lipids that contain saturated alkyl chains with higher transition temperatures are excluded from those that contain unsaturated chains with a lower transition temperature, sometimes below 0 °C, then organizing an ordered, less fluid, liquid phase. Phosphatidylcholine is the major membrane glycerophospholipid, present in a large number of molecular species, differing in the lipid moiety. Dipalmitoylphosphatidylcholine is the main glycerolipid in DRM, and it is highly enriched in saturated species.[214,215] Palmitic and stearic acid are very abundant in glycosphingolipids, and over 60% of total membrane glycosphingolipids are inside the lipid domain fractions, thus resulting in highly enriched fractions. Complex lipids containing palmitic acid are highly enriched in lipid domain fractions, so palmitic acid is the main complex lipid fatty acid of lipid domains.[215,216] In this rigid environment, cholesterol, which alone has a melting point of 148.5 °C, would find a correct position.

The transition temperature of complex lipids does not necessarily correspond to the melting point of the lipid chains. This, in some cases, could introduce confusion. Differential scanning calorimetry (DSC) is used to determine the transition temperatures of complex lipids. But the value is strictly related to the type and size of aggregate formed in solution. Amphiphilic compounds form in solution aggregates of different type and size according to their geometrical properties, which are dependent on both the hydrophilic and hydrophobic moieties. These aggregates range from small, quite spherical micelles that are unfavor-

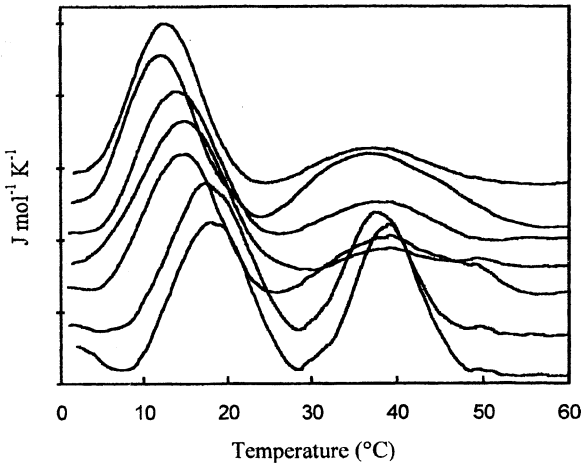

Figure 1. Differential scanning calorimetric runs for GM1 micellar solutions. Dried GM1 was dissolved and maintained at 17, 30, 35, 40, 45, 50, and 60 °C. Analyses were from 2 °C and are displayed starting from the bottom with the solution at lower temperature. The interval between tics corresponds to 200 J/(mol K). Reprinted with permission from ref 217. Copyright 1999 American Chemical Society.

able to chain ordering to large vesicles that instead favor chain ordering (Table 5). Thus, differences in the complex lipid transition temperatures can be observed in the case of aggregates that can largely differ in curvature.[216−219] GM1, which contains stearic acid and a sphingosine with 18 carbons, shows a transition temperature at 11.7 °C, which moves to 23.2 °C by addition of two carbons to the sphingosine. Stable removal of water from the hydrophilic head or changes of the oligosaccharide conformation by increasing temperature lead to changes of the surface area occupied by the monomer inserted into the aggregate with changes in surface curvature, that is, aggregate size.[216−219] Thus, the same GM1 species shows a 5 °C decrease of the transition temperature when dissolved at 60 °C instead of 20 °C (see Table 4). In addition to this, in the case of gangliosides, two different transitions have been observed,[216−219] one related to the hydrophobic moiety and the other, at higher temperature, to the oligosaccharide chains. Figure 1 shows the differential scanning calorimetry behavior of GM1 with 36 carbons in the hydrophobic moiety dissolved at different temperatures each yielding a micelle of different size (see Table 5). It is also necessary to consider that when the complex lipid is not homogeneous in the lipid moiety, being a mixture of molecular species differing in lipid structure, frequently the multiple transitions are purely separated and are represented as a large broadened pick.

4.2. The Hydrophilic Head Group of Glycosphingolipids

As a first general consideration, clustering of some components in a membrane system is favored when the membrane lipids show large differences in the geometrical characteristics of their headgroups, clustering being a spontaneous process due to minimization of the interfacial free energy. This is the case for glycerophospholipids and glycosphingolipids, the latter having headgroups that are much more hydrophilic and much more protruding from the membrane surface. Moreover, glycosphingolipids are a very heterogeneous family of compounds with different content of sugar residues (see Tables 1−3), and with the progressive increase of the headgroup complexity, the molecules inside

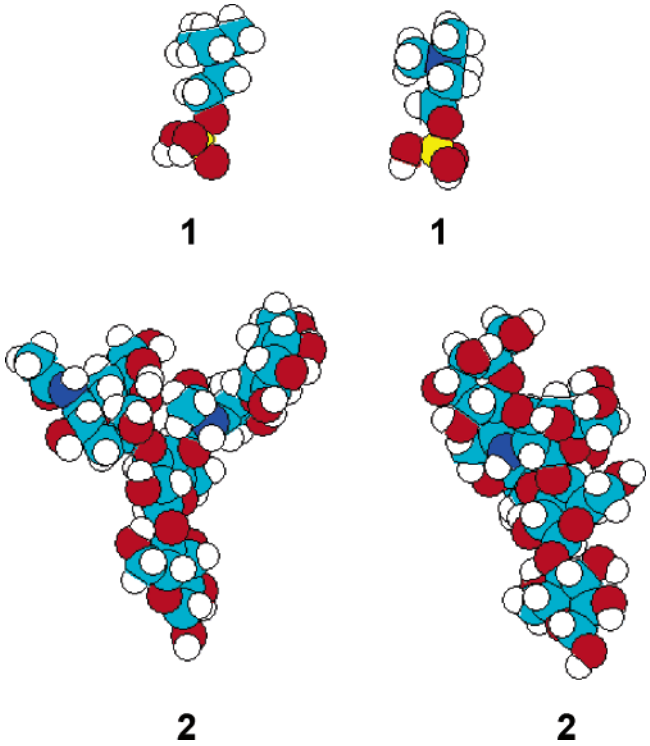

Figure 2. MM2 minimum energy conformation of phosphocholine, **1**, and minimum energy conformation of GM1 ganglioside, **2**, from two view angles according to Acquotti et al.[166]

the membrane require a progressively larger interfacial area to host the hydrophilic headgroup. Then, together with the chemical structure, the dynamic properties and hydration shell of the hydrophilic moiety participate to determine the size of the glycolipid headgroup. Figure 2 shows one of the minimum energy structures of ganglioside GM1,[220] one of the main gangliosides of the nervous system, to compare the size of the carbohydrate chain to that of phosphocholine, the largest group of phospholipids. The large difference in size between glycosphingolipid and glycerolipid headgroups could be considered as a first driving force to the formation of segregation phenomena and of membrane lipid domains. Then these domains will act as a net to segregate cholesterol, which due to its strong hydrophobic character would highly reduce the membrane fluidity, thus creating an optimal zone to block functional proteins.

Within glycosphingolipids, gangliosides (Table 2) have been deeply studied for their geometrical (Table 5) and dynamic (Table 6) properties. Aggregates of natural gangliosides,[124] small aggregates of chemically modified gangliosides that contain a single hydrophobic chain,[221,222] mixed aggregates of gangliosides and dodecylphosphocholine,[220,223−226] monomeric gangliosides,[222,225,227−232] and the free oligosaccharide chains[233] have been used to reveal the relative values of the ganglioside interfacial areas and to suggest small conformational changes deriving from the dynamic properties of the ganglioside oligosaccharide chains. The larger the interfacial area, the more positive the membrane curvature is and the more pronounced the segregation is.

The innermost portion of the oligosaccharide chain of glycolipids is characterized by the disaccharide β-Gal-(1−4)-β-Glc, linked to ceramide, common to all glycolipids with the exclusion of galactosylceramide and GM4, two glycolipids enriched in the myelin.[234] Several likely conform-

Table 6. Glycosidic Torsional Angles ϕ and ψ for Ganglioside Saccharide Sequences[a]

saccharide sequence	linkage	NOE interactions	torsional angles[b] ϕ	ψ
Neu5Acα3Galβ3GalNAcβ	Neu5Ac−Gal	Gal3:Neu5Ac3ax	−160	−20
		Gal3:Neu5AcOH8	−80	+10
	Gal-GalNAc	Gal1:GalNAc2	+30	+14
		Gal1:GalNAc4	+30	−20
		Gal1:GalNAcNH		
GalNAcβ4(Neu5Acα3)Galβ	GalNAc−Gal	GalNAc1:Gal4	+30	+20
		GalNAcNH:Gal2		
	Neu5Ac−Gal	Neu5Ac3ax:Gal3	−160	−20
		GalNAc1:Neu5Ac8		
		GalNAc1:Neu5AcOH8		
		GalNAcOH6:Neu5AcOH9		
GalNAc β4(*Neu5Ac*α8Neu5Acα3)Galβ	GalNAc−Gal	GalNAc1:Gal4	+40	+10
	Neu5Ac−Gal	Neu5Ac3ax:Gal3	+175	+5
		*Neu5Ac3eq:*GalNAc1		
		*Neu5Ac3eq:*GalNAc5		
	Neu5Ac-Neu5Ac	*Neu5Ac3ax:*Neu5Ac6		
		*Neu5Ac6:*Neu5Ac9a		
		*Neu5Ac8:*Neu5Ac9a		
		*Neu5Ac3eq:*Neu5Ac8	+90	+10
Galβ4Glcβ	Gal-Glc[b]	Gal1:Glc4	+5 → +55	−50 → 0
		Gal1:Glc6′		
		Gal1:GlcOH3		
		GalOH2:Glc6′		
		GalOH2:Glc6		
		GalOH2:GlcOH3		
		GalOH2:GlcOH6		
		Gal6:GlcOH3		
Glcβ1Cer	Glc-Cer[c]	Glc1:Cer1	+13	+173
		Glc1:Cer2		
		Glc1:Cer3		
		GlcOH2:CerNH		

[a] Reprinted in modified form with permission from ref 124. Copyright 1994 Elsevier. [b] Linkage fluctuating in a large energy minimum valley with torsional angles ranging (5 → 55, −50 → 0) embracing conformations 1 and 2 of Siebert et al.[225] and conformations (55,0), (5,−30), and possibly (35,−50) of Acquotti et al.[220] [c] Dihedral angles referred to the ^{2}H NMR most likely structure of DTGL from Jarrell et al.[236] This structure was considered by Aubin et al.[238] in analyzing membrane GM3 structure.

ers with similar minimum energy could be predicted,[220,225] but ^{13}C T_1, T_{1r}, and ^{13}C(^{1}H) NOE measurements[223] suggest a reduced mobility for both linkages. The spatial arrangement of these linkages would be better described by fluctuations in a large energy minimum rather than by the sampling of different conformers with short lifetimes. A character of low motional freedom is expected for the Glc residue, fully extended away from the bilayer normal,[235,236] due to the restriction of motion imposed on the saccharide−lipid linkage by the surrounding membrane surface.[237] Glucose, likewise the lipidic portion inserted into the membrane, shows an averaging wobbling motion about the glucosidic bound, the director axis being the bilayer normal, yet the motional averaging effects seem not be large.[238] Instead, in ganglioside GM3, where a sialic acid is linked to the lactose, the α-Neu5Ac-(2−3)-β-Gal linkage has been shown to be very mobile allowing the existence of, at least, two main conformations (Table 6).

In the more complex gangliosides of the ganglioseries such as GM2, GM1, GD1a, and GalNAc-GD1a, the trisaccharide sequence β-GalNAc-(1−4)[α-Neu5Ac-(2−3)-]-β-Gal, as a consequence of several interresidual interactions (Table 6 and Figure 3), is considered a very rigid superunit.[220,223,224,226,231] Any interaction between the C7−C8−C9 side chain of sialic acid, which is in a rigid conformation,[220,227,239,240] and *N*-acetylgalactosamine brings about a strong association between the Neu5Ac and GalNAc units, this association probably being stabilized by a hydrogen bond between the GalNAc amide proton and the Neu5Ac carboxyl group.[232] Figure 3 shows the three-dimensional representation of the β-GalNAc-(1−4)[α-Neu5Ac-(2−3)-]-β-Gal trisaccharide. In

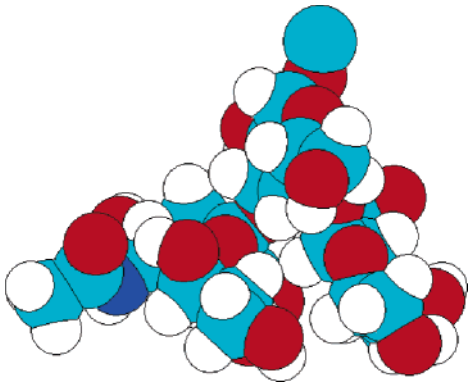

Figure 3. Representation of the trisaccharide rigid unit GalNAc-(Neu5Ac-)Gal according to the torsional angles reported in Table 6.

contrast to this, the addition of galactose to hexosamine makes the disaccharide β-Gal-(1−3)-β-GalNAc very mobile, allowing the existence of, at least, two main conformations of the glycosidic linkage (Figure 4 and Table 6). Thus GM2, carrying only rigid linkages in the outer portion of the oligosaccharide chain, is present only in one preferred conformation, while GM1 carrying the external mobile linkage β-Gal-(1−3)-β-GalNAc is in two and GD1a carrying the external α-Neu5Ac-(2−3)-β-Gal-(1−3)-β-GalNAc is in four (Figure 4 and Table 6). According to the dynamic properties of the glycoside linkages, the ganglioside oligosaccharide moieties are coherently represented by a number of conformers that participate to determine the solid angle occupied by the different ganglioside monomers within

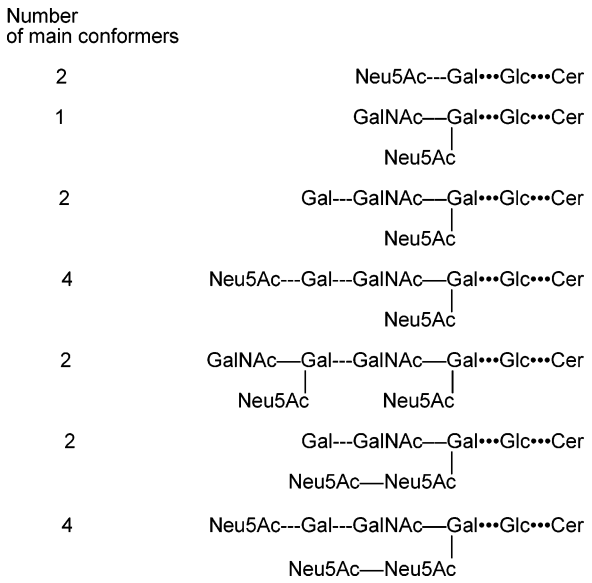

Figure 4. Dynamics of the ganglioside glycosidic linkages as a function of the primary and secondary structure of gangliosides: (—) a single pair of torsional angles; (— — —) two pairs of torsional angles; (•••) flexible linkage.

the surface. It follows that the demand of volume to host the GM1 and GD1a oligosaccharide conformers becomes much higher than that expected to host the chain of GM2 plus one or two additional sugar units. Thus, in GalNAc-GD1a, the addition of a GalNAc to GD1a gives a second β-GalNAc-(1−4)[α-Neu5Ac-(2−3)-]-β-Gal fragment directly bound to the first one, thus reducing the number of conformers from four to two. The four GD1a conformers fill, all together, ca. 1.760 nm^3, calculated as van der Waals sphere volume, vs ca. 1.420 nm^3 occupied by the two GalNAc-GD1a conformers. Thus, the surface area of GalNAc-GD1a is slightly lower than that of GD1a.[231]

By addition of a sialic acid residue to the sialic acid of the β-GalNAc-(1−4)[α-Neu5Ac-(2−3)-]-β-Gal trisaccharide, as in GD1b and GT1b, the interresidual contacts between GalNAc and Neu5Ac linked to Gal are lost, while interactions occur between GalNAc and the external Neu5Ac. According to these constraints, the tetrasaccharide chain β-GalNAc-(1−4)[α-Neu5Ac-(2−8)-α-Neu5Ac-(2−3)-]-β-Gal is arranged in a circle with a hole about 3 Å wide in the center.[230] The internal surface of this hole is highly hydrophobic since seven apolar groups point toward the center. Such a conformation confers a bulkier character to the ganglioside portion closer to the hydrophobic−hydrophilic interface and explains the larger value of surface area of GD1b in comparison to that of its isomer GD1a. It is interesting to note that the solid angle required by the disialosyl chain is wide enough to host an additional sialic acid unit in an external position. In fact, although GT1b carries one more sugar linked to the external galactose unit, it requires the same surface area as GD1b. Instead, when the external sialic acid carboxyl group esterifies the inner sialic acid residue, as in GD1b-lactone, the interresidual interactions between GalNAc and the external Neu5Ac no longer take place, while the compact conformation of the β-GalNAc-(1−4)[α-Neu5Ac-(2−3)-]-β-Gal observed in GM1 and GD1a is restored. This forces a better lining up of the disialosyl chain with the neutral oligosaccharide chain, reducing the angle between the neutral chain and the inner sialic acid axis[230] and explains why geometrical parameters of GD1b-lactone are closer to GD1a than GD1b. A scheme showing the conformational properties of ganglioside glycosidic linkages is reported in Figure 4.

According to their large hydrophilic headgroups and due to the small differences in the oligosaccharide structures, glycosphingolipids are good candidate to stabilize membrane lipid domains and subdomains[241] with positive curvature (Figure 5). The plasma membranes can be considered as a

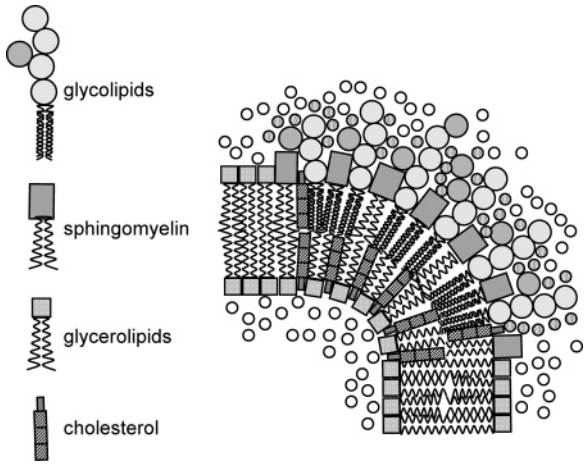

Figure 5. Scheme of a membrane lipid domain enriched in glycosphingolipids. The localization of glycosphingolipids in the outer leaflet of membrane and the large surface area required by the glycosphingolipid monomer to remain inserted into the membrane require a positive curvature of the membrane. In this representation, in agreement with experimental observations,[252,253] cholesterol would be mainly a component of the cytosolic inner leaflet of the membrane.

polymorph system where several lipid organizations can occur and where positive and negative surface curvatures are in sequence. An example of this is represented by *caveolae*,[242] flask-shaped invaginations of the plasma membrane (Figure 6), lacking the clathrin coating and characteristically enriched in proteins of the caveolin family.

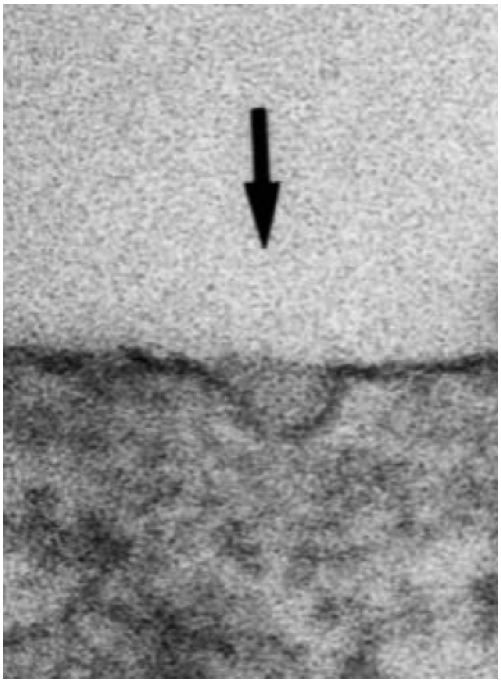

Figure 6. Electron microscopy of MDCK cells. The arrow is in the direction of a caveolar invagination of 60−70 nm diameter.

Caveolae, being flask-shaped, have a negative curvature, thus in principle the presence of glycolipids that belong to the external layer of the plasma membranes is unfavored due to their large hydrophilic headgroups.[124] Data on the absence of gangliosides in these membranes have been reported.[243,244] But the edges of the caveolae must have a strong positive curvature necessary to link up the invagination to the membrane, and this can be obtained with lipids that having very large hydrophilic headgroups show a geometry that is appropriate for a strong positive membrane curvature (Figure 7). This would explain why some reports suggested the presence of gangliosides in caveolae.[245]

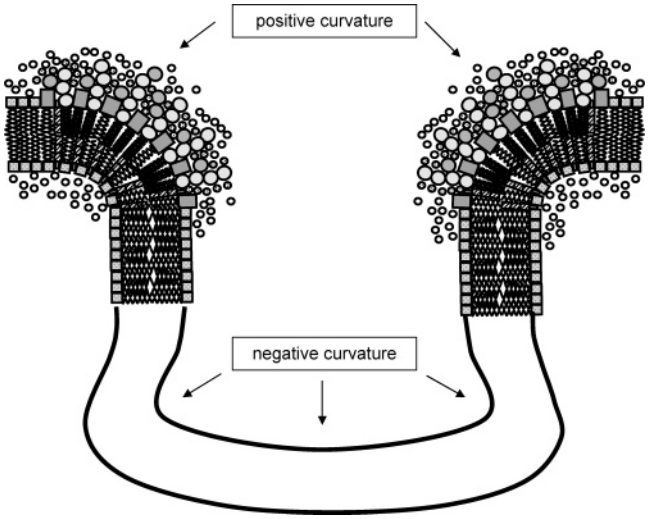

Figure 7. Scheme representing a caveolar invagination. The enrichment of gangliosides at the edges allows the strong positive curvature necessary to link up the negative membrane to the flat membrane environment.

4.3. Side-by-Side Oligosaccharide Interactions and the Carbohydrate Water Shell

Clustering of glycosphingolipids in the membrane system is promoted by their headgroup large sizes, but other events must occur at the membrane surface able to stabilize the segregation. It has been suggested that clustering could be facilitated and stabilized by the formation of carbohydrate−carbohydrate interactions. Nevertheless, while good information is available on the head-to-head interactions,[246] data proving direct side-by-side oligosaccharide interactions are not available. The micellar aggregate of ganglioside was considered a good model to explore this by NMR spectroscopy.[224] In fact at the micelle surface, a single monomer is so close to its neighbor at the level of their headgroups that inter-monomer carbohydrate−carbohydrate interactions could take place and be identified by NOE experiments. Nevertheless, no NOEs were observed between different monomers. In addition, no changes in the carbohydrate chain conformation were detected as an effect of the ganglioside-enriched environment. All this would exclude the presence of significant intermolecular side-by-side interactions capable of altering the "conformational information" carried by the single saccharidic chain of the ganglioside in the micellar model. This could be due to the large amount of water present in the hydrophilic layer.[247] In fact, it should be noted that the actual environment of each monomer at the membrane hydrophilic layer includes solvent.[247] Water is a natural component of the sugar shell being attracted by the hydrophilic character of sugars and by the necessity to avoid repulsion between the negatively charged oligosaccharides.[247] Calculations performed on GM2 micelles[148] indicated a difference of about 5 Å between the dry and hydrated micellar radius. The GM2 micelle is an oblate with ratio of 3.1 between the two axes, and the above value has been determined on an equivalent spherical micelle having the same mass to simplify calculation. This does not allow the number to be known but suggests that several molecules of water are interacting with the oligosaccharide chains. This is in agreement with data from calorimetric studies suggesting that each chain is surrounded by 40−70 water molecules.[248] A strong interaction between water and GM1 sugars, sialic acid, and the inner galactose was observed by NMR.[224] Water bridges between saccharides have been observed in hyaluronan where they were enough strong to determine and stabilize the tridimensional structure of the molecule.[249] Of course these results and considerations would exclude any direct inter-monomer side-by-side carbohydrate interactions at the level of cell membrane but are in favor of a specific role of water in organizing a net of hydrogen bonds able to stabilize the glycosphingolipid clustering.

4.4. The Hydrogen Bond Network at the Lipid–Water Interface

The large differences in headgroup sizes favor clustering of some components in the membrane system; the oligosaccharide−water environment through intermolecular water bridges can stabilize the segregation, but other events much more relevant must occur at the membrane surface to allow and to maintain the membrane lipid domains. This statement derives from the facts that (a) membrane lipid domains exist and are rich in cholesterol, as usual, also in plasma membranes of cells that have a low amount of or are lacking glycosphingolipids,[250] (b) membrane lipid domains very rich in cholesterol and caveolin proteins but with a very low content of neutral glycosphingolipids and no content of ganglioside GM3 have been separated from ganglioside-rich domains starting from the total lipid domain fraction prepared from mouse melanoma B16 cells,[243] and (c) immunolocalization of ganglioside GM3 on the plasma membranes of fibroblasts, where this ganglioside is the main cell ganglioside, showed that it was not a component of the caveolae, membrane invaginations belonging to the DRM fraction.[244] Information on membrane lipid domains in the absence of sphingomyelin is not available. Sphingomyelin is always one of the main sphingolipid components of cell membranes, thus we can say that the ceramide moiety of sphingolipids is always present in the cell membranes, as a moiety of sphingomyelin or as a moiety of both sphingomyelin and glycosphingolipids.

There is a general consensus on a pivotal role played by the water/lipid interface of sphingolipids in promoting the stabilization and formation of membrane lipid domains. The amide group of ceramide is a rigid group comprising six atoms in a planar conformation. The group has a perpendicular orientation toward the axes of the two hydrocarbon chains, the parallel orientation of which is stabilized by the presence of a double bond at position 4−5 of sphingosine.[171,251] Thus, the ceramide moiety can be considered a rigid structure, and addition of glycosphingolipids to cells was shown to reduce the original membrane fluidity.[126] The characteristic presence of a hydroxyl group at position 2 of the amide linkage and of the carbonyl oxygen enables sphingolipids to form hydrogen bonds, acting as hydrogen

bond donors and acceptors at the same time. Therefore, this feature allows sphingolipids to form a stable net of interactions. This is a unique feature of sphingolipids within the all complex lipids. In fact, glycerophospholipids can act only as acceptors of hydrogen bonds, and cholesterol has very limited capacity to form hydrogen bonds. The van der Waals forces between hydrocarbon chains have been estimated to about 2−3 kcal per hydrocarbon chain. The formation of hydrogen bonds at the water/lipid interface contributes with 3−10 kcal to the lipid−lipid interaction. Thus, the orientation of the hydrogen bond donor and acceptor groups of sphingolipids optimal to form lateral interactions and the considerable increase of stability in the lipid association are very good candidates to promote the formation of a membrane rigid zone where a network of hydrogen bond connected lipids are segregated together with cholesterol.

5. Conclusions

Sphingolipids are components of the membranes of all living organism cells. They are abundant in the plasma membranes and show a strong amphiphilic character, being constituted by a two-tail hydrophobic moiety, ceramide, and a very structurally variable hydrophilic headgroup. With ceramide inserted into the external leaflet of the plasma membrane, their hydrophilic headgroups protrude into the extracellular environment, where they can interact with external ligands or with the soluble portion of membrane proteins. Theoretical considerations about the peculiar physicochemical properties of sphingolipids and experimental data suggest that they play an active role in the biogenesis and maintenance of membrane lipid domains, zones of the membrane with reduced fluidity, where proteins involved in processes of cell signaling are segregated and can exert their properties. Moreover, the ability of these molecules to directly interact with membrane proteins suggests that sphingolipids, in general, and glycosphingolipids, in particular, play an important additional role in modulating domain dynamics and functions.

6. Acknowledgment

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