Abstract Phase separation of glycolipids in lipid monolayers and bilayers is of great interest for the understanding of membrane function. The distribution of the ganglioside GM₁ in sphingomyelin (SM)/1-palmitoyl-2-oleoyl-sn-gycero-3-phosphocholine (POPC), SM/1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DOPC) and SM/cholesterol/POC Langmuir-Blodgett (LB) monolayers transferred at 36 mN/m has been studied by scanning force microscopy. Besides lateral organization of the glycolipid in LB monolayers as deduced from topography, material properties have been investigated by phase imaging, pulsed force mode and force modulation microscopy. It was shown that GM₁ preferentially clusters in an ordered lipid matrix, i.e., the SM phase in the case of the SM/POPC and SM/DOPC mixture or in the ordered phase of POPC/SM/cholesterol monolayers. At higher local concentrations, three-dimensional protrusions enriched in GM₁ occur, which may represent a precursor for the formation of micelles budding into the aqueous subphase. Electronic supplementary material to this paper can be obtained by using the Springer Link server located at http://dx.doi.org/10.1007/s00249-002-0232-4.

Keywords Glycolipids · Gangliosides · Monolayers · Membranes · Scanning force microscopy

Introduction

The lateral distribution of receptors in lipid bilayers is of pivotal interest for the understanding of ligand-receptor interactions at the cellular membrane, the structure-function relationship of endocytosis, membrane trafficking and signal transduction. In particular, gangliosides, negatively charged ceramide-based glycolipids bearing at least one sialic residue, have been recognized as important receptor lipids for bacterial toxins, lectins, initial docking of virus capsids and also as modulating compounds for other receptors such as the insulin receptor and integrins, which are responsible for cell-substrate interaction (Fishman and Brady 1976; Habermann and Dreyer 1986; Hakomori 1981; Holmgren et al. 1980; Janshoff et al. 1996, 1997; Singh et al. 2000; Tamm et al. 1996). For instance, the monosialoganglioside GM₁ is known to be the natural receptor for cholera toxin. Despite their abundance, little is known about the lateral structural organization of glycolipids in the outer leaflet of the biological membrane. An increasing amount of convincing evidence has been collected that points towards the formation of ordered microdomains in the outer leaflets of cellular membranes that are enriched in sphingolipids and cholesterol, thus providing the prerequisite for the organization and clustering of receptor molecules within the membrane (Brown and London 1998a, 1998b, 2000; Dietrich et al. 2001; Vyas et al. 2001). Treatment of the plasma membrane with detergents results in the formation of a detergent-resistant network referred to as DIGs – detergent insoluble membrane fractions (Brown and London 2000; Fivaz et al. 2000; Simons and Ikonen 1997). These submicron domains, rafts, display physical properties which can be identified with the liquid ordered phase (Lₒ phase). The Lₒ phase is an intermediate between the gel phase and the liquid disordered phase, showing significant higher lateral mobility than lipids in the gel phase along with more ordered chains (Brown and London 2000).

Model membranes of different kinds such as liposomes, solid-supported bilayers and monolayers at the air-water interface have been used throughout the literature to investigate phase separation phenomena, avoiding the state of complexity met in biological membranes. Although the organization of gangliosides in model membranes has been extensively studied by a
variety of different techniques, there is still lack of consistency, which may partly be attributed to a different scale, i.e. spatial resolution, labeling techniques, interference from probes and preparation of the membranes (Yuan and Johnston 2000 and references therein). Recently, scanning probe techniques have added a significant amount of invaluable information about phase separation in mono- as well as bilayers systems (Dufrene and Lee 2000; Ross et al. 2001; Yuan and Johnston 2000, 2001). For instance, Le Grimelloc and co-workers performed a thorough study on the domain formation of various monolayers consisting of sphingomyelin (SM), cholesterol and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), modeling the outer leaflet of the renal brush border membrane (Milhiet et al. 2001). The authors found phase separation in the POPC/SM monolayers (liquid expanded/liquid condensed, LE/LC) up to a content of 33 mol% cholesterol. The persistent lateral heterogeneity is probably due to the preferential interaction of cholesterol with SM, as pointed out by Slotte (1999).

Vie et al. (1998) showed that ganglioside GM1 is enriched in the liquid condensed phase of mixed monolayer films of DPPC and DOPC. Further confirmation that gangliosides preferentially distribute in the ordered phase has been provided recently by Yuan and Johnston (2000). The authors observed clustering of GM1 in the center of the LC phase and at the boundary of the LC domains. GM1-enriched domains could be identified by an increased height due to the bulky headgroup of GM1 and clustering was attributed to weak carbohydrate interactions. In contrast, Mou et al. (1995) found no segregation of GM1 in phosphatidylcholine bilayers by scanning force microscopy (SFM) using cholera toxin as a label.

In this paper we investigate, for the first time, phase separation and material properties of mixed monolayers of SM, cholesterol and POPC or DOPC supplemented with a low content of GM1 (0.2 mol%) by means of SFM, providing high-resolution spatial information about topography, elasticity and adhesion on a nanometer scale. The study provides convincing evidence, by combining topography information with phase imaging and force modulation microscopy, that GM1 is indeed preferentially distributed in more ordered membrane regions such as the SM-enriched phase, but also forms domains enriched in GM1 within the ordered matrix itself. Furthermore, we found evidence for the formation of GM1-enriched micelles budding into the water subphase, as predicted by McConnell (Radhakrishnan and McConnell 2000).

**Materials and methods**

**Materials**

POPC, DOPC and SM from brain were purchased from Avanti Polar Lipids (Alabaster, Ala., USA) and used without further purification. 45.5% of the SM consists of 18:0 fatty acids, 23.3% of 24:0, 7.2% of 22:0, 5.1% of 20:0, 1.7% of 16:0 and 6.3% were unsaturated 24:1 chains. Cholesterol, chloroform, ganglioside GM1 and methanol were purchased from Sigma-Aldrich (Dreieich, Germany).

**Film balance measurements**

Surface pressure-area isotherms were obtained at 20 °C on a Wilhelmy balance (Riegler and Kirstein, Golm, Germany) with an operational area of 144 cm². Lipid monolayers were spread on a subphase containing ultrapure water. The organic solvent was allowed to evaporate for 10 min before the film was compressed at a rate of 2.8 cm²/min. For the preparation of Langmuir-Blodgett (LB) films a Wilhelmy balance equipped with a 25 ml Teflon trough (15.4 cm x 2.5 cm) and a diaphragm device was used. LB films for SFM measurements were deposited on freshly cleaved mica sheets. After 10 min, the monolayer was compressed to a surface pressure of 36 mN/m at a rate of 1.79 cm²/min and equilibrated for 30 min. The lipid film was deposited onto the substrate with a pulling speed of 0.7 mm/min while maintaining the surface pressure to a constant value of 36 mN/m.

**Scanning force microscopy**

Surface images of the LB monolayers were obtained using a Nanoscope IIIa (Bioscope) scanning force microscope (Digital Instruments, Santa Barbara, Calif., USA) operating in contact mode, tapping mode, force modulation (FM, Jourdan et al. 1999) and pulsed force mode (PFM, WiTec, Ulm, Germany) (Magonov et al. 1997; Rosa-Zeiser et al. 1997). Microfabricated silicon nitride tips (NP-S, Digital Instruments) with an approximate tip radius of 5–20 nm and a nominal spring constant of 0.06–0.56 N/m were used as purchased for force modulation, pulsed force and contact mode microscopy. For tapping mode measurements, silicon tips with an approximate resonant frequency of 300 kHz and an approximate force constant of 48 N/m were purchased from Nanosensors (Wetzlar-Blankenfeld, Germany).

**Results and discussion**

The motivation for investigating LB monolayers composed of DOPC/SM (80:20) or POPC/SM (80:20) is based on the fact that most plasma membranes exhibit 10–20 mol% SM, in which most of the lipid is located in the outer leaflet. According to Brown and London (2000), a lipid composition of POPC/Chol/SM of 2:1:1 should result in coexistence of the liquid disordered and the liquid ordered phases. This way we can decide if GM1 is preferentially distributed in an ordered or a disordered matrix.

Monolayers composed of DOPC/SM (80:20) or POPC/SM (80:20) transferred to mica at 36 mN/m display typical phase separation, in which the higher phase (lighter) consists of SM in the LC phase and the lower phase of DOPC is in the LE phase up to collapse of the monolayer at high pressure. Figure 1A shows a representative SFM image (topography) obtained from tapping-mode imaging of a LB monolayer of DOPC/SM (80:20), while Fig. 1B shows the phase information, revealing that the higher regions corresponding to the SM phase exhibit a larger phase shift. This might be interpreted as a higher elastic modulus for the SM domains. This is inferred from the chosen setpoint (A set/A free between 0.4 and 0.75), indicative of moderate tapping, which provides image contrast mainly based on