PROBING LIPID DOMAINS IN MODEL MEMBRANES AND IN MAMMALIAN CELLS

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‘All we have to decide is what to do with the time that is given us.’

-J. R. R. Tolkien (1966) The Fellowship of the Ring-

CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following publications which are referred to in the text by their Roman numerals:

I. Koivusalo, M., Haimi, P., Heikinheimo L., Kostiainen R., Somerharju P. (2001) Quantitative determination of phospholipid compositions by electrospray mass spectrometry. Effects of acyl chain length, unsaturation and lipid concentration on instrument response. *J.Lipid Res. 42: 663-672*

II. Blom T.S.*, Koivusalo M.*, Kuismanen E., Kostiainen R., Somerharju P., Ikonen E. (2001) Mass spectrometric analysis reveals an increase in plasma membrane polyunsaturated phospholipid species upon cellular cholesterol loading. *Biochemistry 40: 14635-14644*
*** = equal contribution**

III. Koivusalo, M., Alvesalo, J., Virtanen, J., Somerharju, P. (2004) Partitioning of pyrene-labeled phospho- and sphingolipids between ordered and disordered membrane domains. *Biophys. J. 86 (2): In Press*

In addition, some unpublished data is presented.

ABBREVIATIONS

BB-SM	bovine brain sphingomyelin
BHK	baby hamster kidney
BODIPY-SM	*N*-(4,4-difluoro-5,7-dimethyl-4- bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingomyelin
CHO	chinese hamster ovary
CTxB	cholera toxin subunit B
DHE	dehydroergosterol
$DiIC_n$	1,1'-*n*-3,3,3',3'-tetramethylindocarbocyanine (*n* = number of aliphatic carbons)
$DiOC_n$	3,3'-dihexadecyloxacarbocyanine tetramethylindocarbocyanine (*n* = number of aliphatic carbons)
DRM	detergent resistant membrane
E	excimer
ESI-MS	electrospray ionisation mass spectrometry
GalCer	galactosylceramide
γ-CD	γ-cyclodextrin
GlcCer	glucosylceramide
GPI	glycosylphosphatidylinositol
HPLC	high performance liquid chromatography
LacCer	lactosylceramide
LBPA	lysobisphosphatidic acid
l_c	liquid-crystalline
l_d	liquid-disordered
l_o	liquid-ordered
M	monomer
MDCK	Madin-Darby canine kidney
NBD-SM	6-((*N*-(7-nitrobenz-2-oxa-1,3- diazol-4-yl)amino)hexanoyl)sphingomyelin
NPA	Niemann-Pick A
NPC	Niemann-Pick C
PA	phosphatidic acid
PC	phosphatidylcholine

PE	phosphatidylethanolamine
PI	phosphatidylinositol
PS	phosphatidylserine
Pyr_n	pyrenylacyl (*n* = number of aliphatic carbons in the pyrenylacyl chain)
SCD	stearoyl-coenzyme A desaturase
SLSD	sphingolipid storage disease
s_o	solid-ordered
SM	sphingomyelin
SMase	sphingomyelinase
TGN	*trans*-Golgi network
T_m	gel-to-fluid main phase transition temperature
TLC	thin layer chromatography
TNP-LPE	trinitrophenyl-lysophosphatidylethanolamine
VSV	vesicular stomatitis virus
7-SLPC	1-palmitoyl-2-(7-doxyl)-stearoyl-phosphatidylcholine

ABSTRACT

Mammalian cell membranes consist of a vast variety of glycerophospholipids, sphingolipids and cholesterol. The cell membranes have been commonly considered to be fluid, homogenous bilayers. Recently, however, it has been proposed that lipids in some cellular membranes are not randomly distributed, but instead show local heterogeneity due to segregated microdomains. The microdomains have been named 'rafts' and they are thought to form an ordered phase rich in sphingolipids, saturated phospholipids and cholesterol, as well as certain proteins. Rafts have been associated with many important biological processes, such as protein and lipid sorting, signal transduction, endocytosis and cell migration. Despite the highly active research, however, the presence and properties of rafts in cellular membranes remain to be unambiguously demonstrated.

To study the lipid composition and properties of membrane domains, highly sensitive analytical tools are needed. Electrospray ionisation mass spectrometry (ESI-MS) is a very sensitive and specific method for quantitative determination of lipid compositions. Another useful method to study membrane domains is to use fluorescent lipid analogues with properties similar to endogenous lipids of cells, which provide a means to study domains and lipid trafficking in living cells. In this thesis, these two analytical methods were developed further, and used on mammalian cells to study the composition and properties of membrane domains.

First of all, several factors affecting quantitative analysis of phopholipid compositions by ESI-MS were examined. Phospholipid acyl chain length and unsaturation, polar headgroup and total lipid composition were found to have a marked effect on the instrument response. By including several internal standards for each lipid class, these factors could be reliably corrected for and quantitative data on cellular phospholipid composition were obtained. ESI-MS was used to analyse lipid composition of viruses budded from ordered and disordered plasma membrane domains of human fibroblasts. The ordered plasma membrane domains ('rafts') were enriched in glycosphingolipids in comparison with the disordered domains ('non-rafts'). The influence of chronic and acute cholesterol loading on phospholipid composition of the plasma membrane domains as well as of whole cells was also studied. Cholesterol loading increased the content of

polyunsaturated acyl species in phospholipids of both the plasma membrane and whole cells, which may reflect a regulatory mechanism to maintain an appropriate level of membrane fluidity against the membrane stiffening effect of cholesterol.

Second, we characterised how different fluorescent, pyrene-labeled phospho- and sphingolipids partition between ordered and disordered domains in model bilayers. The partitioning was strongly affected by the length of the labeled acyl chain; the long-chain analogues favored the ordered phase, whereas the short-chain analogues partitioned preferably into the disordered phase. The information on how pyrene-labeled lipids partition between domains in a model system allows their use in studying domain organisation in living cells. For this purpose, pyrene-labeled sphingomyelins with different acyl chain lengths were introduced into human fibroblasts with the aid of γ-cyclodextrin. The domain partitioning of the pyrene lipids in cells was assessed by their detergent insolubility and found to be similar to the partitioning in model membranes. The cellular distribution was studied with fluorescence microscopy. Since pyrene-labeled lipids with different domain-partitioning preferences can be introduced into cells, these analogues are useful tools in studying domain properties and lipid trafficking in cells.

INTRODUCTION

The lipids in the membranes of mammalian cells consist of glycerophospholipids, sphingolipids and cholesterol. The most abundant lipids are unsaturated glycerophospholipids which form a fluid phase, termed as liquid-disordered (l_d) or liquid-crystalline (l_c) phase [1]. The organisation of cellular lipids into membranes has commonly been based on the fluid-mosaic model according to which the lipids form a homogeneous matrix [2]. Recently, however, a different view of cellular membranes has emerged as it has been proposed that cellular lipids can segregate into microdomains that have often been named rafts [3-6]. Much of the data on rafts have been derived from biochemical studies of detergent-resistant membranes (DRMs) that can be isolated from cell lysates by non-ionic detergents at 4 °C [3, 7]. It is believed that rafts consist of cholesterol and lipids with saturated acyl chains, such as sphingolipids. These lipids can form a phase known as liquid-ordered (l_o) phase which separates from l_d phase due to its more ordered character [8, 9]. The presence of l_o phase has been suggested to contribute to the detergent-insolubility of rafts [4, 10-12]. Enrichment of certain proteins in rafts has been reported, these proteins include glycosylphosphatidylinositol (GPI)-anchored proteins, doubly acylated proteins and some transmembrane proteins [3, 4, 7, 10, 13-18]. Nevertheless, conclusive evidence for the existence of rafts in cellular membranes is still lacking.

Rafts have been suggested to have many important functions in cells. They can mediate lipid and protein sorting in the Golgi membranes [3, 19] and also in endosomes [20-22]. Endosomal raft lipid and protein sorting is defective in certain lysosomal storage disorders, such as Niemann-Pick C (NPC) disease, in which cholesterol and sphingolipids accumulate in late endosomes/lysosomes [21, 23]. Another essential role of rafts in cells may be formation of platforms for signaling events, as they confine proteins involved in a signal transduction event into the same microdomain [3, 4, 24]. Raft-mediated signal transduction could play a role, for instance, in hematopoietic cells during immune response [4, 25], in mediation of apoptotic signals [26] and in infection of cells by bacteria, viruses and parasites [27-29]. In addition, rafts can serve as docking sites for certain pathogens and toxins [27-29], and they may be involved in cell migration in response to chemoattractants [30].

Maintenance of a proper level of fluidity inside as well as outside rafts is important for membranes because fluidity determines the physicochemical properties of the membrane. Membrane fluidity is determined mainly by the degree of phospholipid acyl chain unsaturation and the level of cholesterol [31, 32]. An increase in the degree of phospholipid acyl chain unsaturation increases membrane fluidity [33]. In contrast, an increase in cholesterol content increases the order of phospholipids in the fluid, l_d phase, therefore decreasing the fluidity of membranes [8, 31, 32, 34, 35]. As a result, a physiological cholesterol/phospholipid ratio is important for cells to maintain proper membrane fluidity [31, 32]. For this purpose, normal mammalian cells tightly regulate cholesterol synthesis and uptake [36] and synthesis of unsaturated fatty acids [37]. Synthesis of unsaturated fatty acids is highly regulated at the level of Δ9 desaturase (stearoyl-CoA desaturase, SCD), which is the rate-limiting enzyme in the synthesis of monounsaturated fatty acids and whose preferred reaction is formation of oleoyl(18:1)-CoA from stearoyl (18:0)-CoA [37].

To study the lipid composition of membranes and membrane domains, sensitive analytical methods are required. Traditionally, lipid compositions have been determined by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) [38] for which rather large amounts of sample material are required. Several chromatographic and enzymatic steps are also often required, which makes analyses complicated. Electrospray ionisation mass spectrometry (ESI-MS) offers a highly sensitive and specific tool to quantitatively determine the total lipid composition of small amounts of membrane samples. ESI is a ‘soft’ ionisation method, which has been successfully applied to quantify hundreds of chemically distinct lipids in cells and cellular membranes [39-42].

Widely used techniques to study domain properties in model membranes and domain-based lipid trafficking within living cells are fluorescence spectroscopy and microscopy. For this purpose, fluorescent lipid analogues are essential. To date, most experimental evidence of lipid sorting based on differential partitioning into ordered domains in cells has largely been derived from studies examining trafficking of lipid analogues with a polar fluorophore attached to a short acyl chain [43]. However, due to the polarity of the fluorescent moiety, the properties of these analogues may not reflect the properties of natural lipids [44-46]. Therefore, fluorescent

analogues more appropriately mimicking endogenous lipids should be employed to obtain relevant information on lipid domains.

The aim of this thesis was to further develop the aforementioned analytical tools, and to implement them to study the composition and properties of lipid domains in model membranes and in cells.

REVIEW OF THE LITERATURE

1. ORGANISATION OF LIPIDS IN CELLULAR MEMBRANES

1.1 Membrane lipids and their distribution between different organelles

Mammalian membranes contain an enormous variety of different glycerophospholipid and sphingolipid species. The glycerophospholipids are characterised on the basis of their polar headgroup, the most abundant one is phosphatidylcholine (PC). Other major classes are phosphatidylethanolamine (PE), phosphatisylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA) and phosphatidylglyceol (PG). In glycerophospholipids the fatty acid in *sn*-1 position of the glycerol backbone is usually saturated, whereas the fatty acid in *sn*-2 position is unsaturated. The backbone in sphingolipids is the sphingoid base, which in mammals usually is 4-*trans*-sphingenine or 'sphingosine' [47]. The sphingosine is *N*-acylated with a long-chain, most often saturated, acyl chain to form a ceramide. Sphingolipids are often divided into phosphosphingolipids and glycosphingolipids based on their headgroup. In mammals the phosphosphingolipid is sphingomyelin (SM), which contains a phosphocholine head-group like PC. Glycosphingolipids are classified according to the structure of the carbohydrate head-group. Neutral glycosphingolipids contain uncharged sugars giving rise to e.g. galactosylceramide (GalCer), glucosylceramide (GlcCer) and lactosylceramide (LacCer). Acidic glycosphingolipids contain ionised functional groups such as sulfate in sulphatide, or charged sugars such as sialic acid (*N*-acetyl neuramic acid) in gangliosides.

Many lipids are heterogeneously distributed among membranes surrounding cellular organelles. The plasma membrane is enriched in unesterified cholesterol [48, 49]. Depending on the cell type and the assay method used, plasma membrane has been reported to contain up to ~60-90% of the total cellular cholesterol [50-52]. SM is also highly enriched in plasma membrane [50, 52, 53]. In addition to cholesterol and SM, the majority of cellular glycosphingolipids [54, 55], and PS [49, 52] also reside in plasma membrane. Polarised cells, such as epithelial cells, have two distinct plasma membrane domains, apical and basolateral domains, which are separated by tight

junctions. Apical domains are rich in (glyco)sphingolipids and basolateral domains in PC in Madin-Darby canine kidney (MDCK) cells [56, 57].

The endoplasmic reticulum (ER), the site of cholesterol synthesis, has a very low cholesterol and sphingolipid content [49, 54]. The cholesterol and sphingolipid contents of the Golgi compartment, on the other hand, are intermediate between those of the plasma membrane and the ER [54, 55]. Also when Chinese hamster ovary (CHO) cells were labeled with a fluorescent cholesterol analog, dehydroergosterol (DHE), this cholesterol analogue became enriched in the *trans*-Golgi network (TGN) [58]. Thus, there seems to be an increasing concentration of cholesterol and sphingolipids along the secretory pathway from ER to plasma membrane [59]. This increasing concentration may be achieved by excluding cholesterol and sphingolipids from vesicles transporting components from the Golgi to the ER [60].

Endosomal compartments have been found to contain cholesterol and SM concentrations similar to those of plasma membrane [61]. In particular, recycling endosomes in the polarised MDCK cells were enriched in cholesterol, SM and PS [62]. Also DHE was reported to localise significantly to recycling endosomes in CHO cells [58, 63]. In contrast, late endosomes were shown to be depleted in SM, PS and cholesterol [64, 65].

Cells obtain different lipids either by endocytosis from outside of the cell or by *de novo* synthesis. Synthesis of phospholipids and cholesterol takes place in ER. Sphingolipid synthesis starts in ER with assembly of ceramide and continues in Golgi by synthesis of GlcCer, the precursor of higher sphingolipids, and by synthesis of SM [47, 55]. Since cell organelles have specific lipid compositions, selective tranport of the endocytosed and synthesised lipids are needed between different compartments. Lipid transport between cellular membranes can occur by a) spontaneous diffusion between cytosolic leaflets of cellular membranes, b) lipid transfer proteins, c) lateral diffusion via membrane (hemi)fusion sites or d) vesicular transport [66]. The major routes of vesicular transport are those of endocytosis and exocytosis. At each vesicle budding event specific membrane components must be included or excluded from the budding vesicle to direct compounds to their appropriate destination. This process is called membrane sorting.

1.2 Transbilayer asymmetry

In addition to the enrichment of certain lipids, transbilayer lipid asymmetry is another important feature of various cellular membranes. For instance, PS, PE and possibly PI as well are predominantly located in the inner leaflet, while PC, SM and glycosphingolipids are enriched in the outer leaflet of the plasma membrane [67-69]. Transbilayer distribution of cholesterol is still controversial. Because cholesterol has been proposed to preferentially associate with SM compared to other phospholipids [70], one would expect that it prefers the outer leaflet of plasma membrane [71]. However, not all studies show a specific affinity between SM and cholesterol [72-74], and there is experimental evidence also suggesting that cholesterol can be predominantly found in the inner leaflet [75].

There are various ways how lipids can attain the asymmetric distribution of the plasma membrane. For complex glycosphingolipids, the outer leaflet localisation is in line with the fact that they are synthesised on the lumenal side of the Golgi membrane, the topological equivalent of the extracellular leaflet of the plasma membrane [55, 71]. To facilitate translocation (i.e. flip-flop) of polar lipids across membranes specific transport proteins exist in cells. Flippases are proteins, which mediate ATP-independent, bi-directional transbilayer movement of lipids but are incapable of accumulating lipids in one leaflet [76]. For instance, GlcCer, the precursor of more complex glycosphingolipids, is synthesised on the cytosolic surface of early Golgi. Therefore, for the synthesis of higher glycosphingolipids, GlcCer has to be moved across the Golgi membrane to the lumenal side [77] possibly by a GlcCer flippase [76]. Translocases, on the other hand, facilitate ATP-dependent unidirectional movement of lipids against a concentration gradient in a membrane [76]. Aminophospholipid translocase in plasma membrane selectively translocates PE and PS inward from the exoplasmic leaflet to the cytoplasmic leaflet [69, 71]. An aminophospholipid translocase has been localised also to Golgi, raising the possibility that the lipid asymmetry may be established already in the Golgi [78]. There are also translocases which can move lipids from the cytoplasmic leaflet to the exoplasmic leaflet of the plasma membrane, such as some members of the ATP-binding cassette (ABC) transporter family. For instance, it was shown that multidrug resistance (MDR) 3 P-

glycoprotein specifically mediates transport of short-chain PC analogues from the cytoplasmic to the exoplasmic side, whereas MDR 1 P-glycoprotein transported a variety of lipid analogues [79, 80].

Lipids can also spontaneously translocate across a bilayer. In this process the lipid has to traverse the hydrophobic bilayer interior, therefore, neutral lipids like cholesterol, ceramide and diacylglycerol translocate rapidly, whereas spontaneous transbilayer movement is slow for lipids with a large or charged head-group, such as glycosphingolipids and phospholipids [69, 71].

1.3 Lateral distribution

1.3.1 The raft model

According to the classical fluid-mosaic model of cellular membranes by Singer and Nicolson, phospholipids and proteins are randomly distributed in a membrane and membranes exist in l_d phase [2]. However, a concept of lateral heterogeneity of lipids in biological membranes was presented early on [81-84]. The best understood example of lateral heterogeneity is phase separation into gel and l_d phases. Each lipid has its characteristic gel-to-fluid main phase transition temperature (T_m) defined for one-component systems. Below T_m, the lipid bilayer is in gel, or solid-ordered (s_o) phase, characterised by highly ordered acyl chains and translational order in the bilayer plane (**Fig. 1A**). Above T_m, the bilayer is in the fluid, l_d phase with acyl chains disordered and loosely packed (**Fig. 1A**). Unsaturated glycerophospholipids generally have a very low T_m, whereas sphingolipids and other commonly saturated lipids tend to have a high T_m [1, 12, 33, 82]. If two lipids with different T_m are mixed in a model membrane and kept at a temperature between the T_m of the two lipids, this binary mixture separates into gel and fluid phases [84-91].

Since eukaryotic plasma membranes are mixtures of low-T_m glycerophospholipids and high-T_m sphingolipids, it was thought that plasma membrane could be composed of co-existing gel-state sphingolipid domains and fluid-state phospholipid domains [83, 84]. However, gel-state lipids are not usually found in cell membranes [12, 92]. In addition, the presence of cholesterol in

cellular membranes argued against the presence of lipid domains, because cholesterol tends to eliminate the transition of saturated PC species from gel to l_d phase [93-95] (*see section 2.2*). Subsequent studies indicated, however, that in cholesterol-containing bilayers lipids can exist in l_o phase [8, 34, 35, 96] (**Fig. 1A**), a phase other than gel or l_d phase, which made the idea of domain segregation more relevant.

Most data on domain formation in cellular membranes derives from the findings that a part of cellular membranes is insoluble in non-ionic detergents such as Triton X-100 at +4 °C [3, 7, 91]. Such detergent resistant membranes (DRMs) were first suggested to represent sphingolipid clusters present in erythrocyte membranes before the detergent treatment [97]. In addition to sphingolipids, cholesterol was also shown to be enriched in DRMs [7]. Subsequently, DRMs have been isolated from various mammalian cells [4]. A functional role for membrane domains was proposed by Simons and van Meer, who argued that sphingolipid-rich domains in Golgi are involved in sorting of lipids and proteins to the apical plasma membrane domains of polarised cells [57]. Subsequently, these Golgi membrane domains were shown to associate with DRMs [7, 17].

Simons and Ikonen reintroduced the concept of lateral heterogeneity in cellular membranes by presenting 'the raft model' of biological membranes [3]. According to this model, sphingolipids and cholesterol segregate in the cellular membranes to form microdomains termed rafts, which are detergent-insoluble. According to the model, rafts are formed by lateral association of sphingolipids in the exoplasmic leaflet, and voids between sphingolipid molecules are filled by cholesterol molecules (**Fig. 1B**). Various studies proposing that SM may specifically interact with cholesterol support this view [70, 98]. In addition, recent computer simulation studies show intramolecular hydrogen bonding and an increased ordering of hydrocarbon chains in SM molecules [99, 100], which may also contribute to phase separation of sphingolipids from the more fluid phospholipids in membranes. Even though the nature of phospholipids in the cytoplasmic leaflet is largely unknown, the two leaflets in membrane domains probably interact with each other (*see section 4.1*). The current view is that rafts are dynamic and unstable structures with estimated sizes from a few molecules to hundreds of nanometers, and they have the ability to form larger patches under certain conditions [3, 5, 24, 101]. Rafts have been

reported to be enriched in GPI-anchored proteins in the exoplasmic leaflet [7, 10, 13], acylated tyrosine kinases in the cytosolic leaflet [4, 14-16], as well as some transmembrane proteins [3, 4, 17, 18]. Nevertheless, the presence of rafts in cellular membranes has not yet been unambiguously demonstrated.

1.3.2 The superlattice model (regular distribution model)

It has been proposed that different lipid classes could adopt regular (superlattice-like) rather than random distributions in mixed lipid bilayers [102, 103]. Based on this superlattice model there are only a limited number of allowed, 'critical' concentrations for each component. Superlattices have many interesting features. First, they are thought to form because they represent the energetically most favourable packing of the membrane components. Second, superlattices do not cover the whole membrane area at any time but are in equilibrium with randomly arranged domains as well as superlattices with a different composition. Third, despite a high degree of local order, superlattices have no long-range order.

There is significant experimental evidence for superlattices in model membranes. Studies on fluorescent, pyrene-labeled lipids in model membranes have shown that the ratio of excimer to monomer fluorescence intensities that is proportional to the pyrene lipid collisional frequency, does not increase smoothly with pyrene lipid mole fraction, but kinks or dips were observed at particular pyrene lipid mole fractions coinciding with critical mole fractions predicted by the superlattice model [104, 105]. Also sterols in phospholipid bilayers seem to adopt regular distributions. Evidence for this was first shown for a fluorescent sterol analogue, dehydroergosterol [106] and later for cholesterol in cholesterol/PC bilayers [107, 108]. In addition, complementary shapes of lipids may drive superlattice formation, for instance in PE/PC bilayers where PE causes 'packing frustration' in the presence of PC (larger headgroup than PE) [109].

An intriguing implication of the superlattice model is that the 'critical' compositions may play a role in regulating the lipid compositions of natural membranes [102, 103]. This possibility is based on the finding that the phospholipid composition of mammalian red cells agrees closely

with the 'critical' compositions of the superlattice model [110]. Interestingly, superlattice formation could also drive the segregation of the cellular organelles, e.g. between the ER and the Golgi membranes. In this case the boundary between these two organelles could be formed by different cholesterol superlattices [102]. Notably, the superlattice model and the raft model are not mutually exclusive. For instance, the lipid composition of rafts could be determined by the superlattice principle [102, 103].

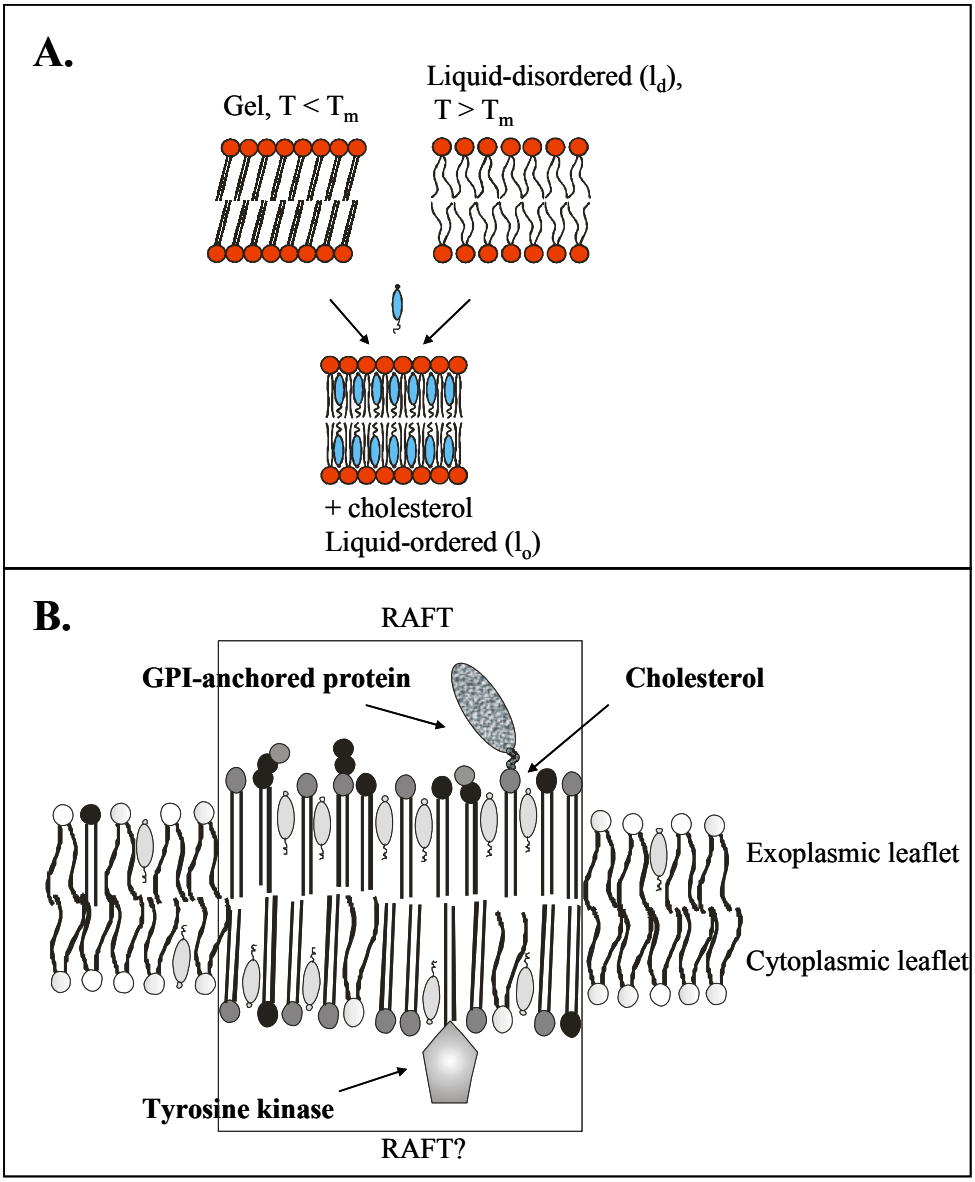

Figure 1. (A) Different phases that can exist in a bilayer. (B) A model of a cellular membrane containing sphingolipid/cholesterol-rich l_o microdomains.

2. DOMAIN FORMATION IN MODEL MEMBRANES

2.1 Segregation into gel (s_o) and liquid-disordered (l_d) phases

Separation of lipids into gel (=solid-ordered, s_o) and fluid (l_d) phases has been observed in one-component bilayers in the temperature region of the main phase transition of saturated PC species [87]. In binary mixtures of PC, species with different acyl chain lengths or different degrees of unsaturation separate into co-existing gel and l_d phases at temperatures between the T_m of the two lipids [12, 84-90]. In addition, binary systems consisting of a high-T_m lipid (either PC or SM) and a spin-labeled PC species also show phase separation because spin-labeled PC species contain a bulky nitroxide-bearing doxyl group in their acyl chain, and therefore, they have a low T_m similarly to unsaturated PC species [111-113]. Spin-labeled lipids quench fluorophors which enables their use in studying domain partitioning of different fluorescently labeled lipids [113-115].

Different lipid classes also show non-ideal mixing. Binary bilayers consisting of a high-T_m SM and a low-T_m PC have been shown to segregate into gel and l_d phases [116, 117]. Also certain glycosphingolipids form lateral clusters in PC bilayers [84, 118-121]. These observations led to a concept that plasma membrane may be composed of co-existing gel-like sphingolipid domains and fluid phospholipid domains [84]. Phospholipids with different polar headgroups can also segregate into gel and l_d phases [86, 88].

2.2 Segregation in bilayers containing cholesterol

Cholesterol changes dramatically the phase behaviour of lipids. In binary mixtures of cholesterol and saturated PC species, cholesterol can suppress the ability of saturated PC species to separate into distinct gel and l_d phases [93-95, 122]. This effect of cholesterol is based on its ability to control the fluidity of phospholipid acyl chains by decreasing the acyl chain order in gel phase and increasing the order in l_d phase [8, 34, 35, 93, 123]. Recent computer simulation studies have provided further evidence indicating that cholesterol increases the order of saturated phospholipid acyl chains in l_d phase [124, 125]. At the same time, cholesterol can also

induce phase segregation that is different from gel/l_d phase separation. Binary mixtures consisting of a saturated PC species (or SM) and a defined amount of cholesterol contain the cholesterol-rich l_o phase that has characteristics between s_o and l_d phases [8, 34, 35, 96, 99, 123] (**Fig. 2**).

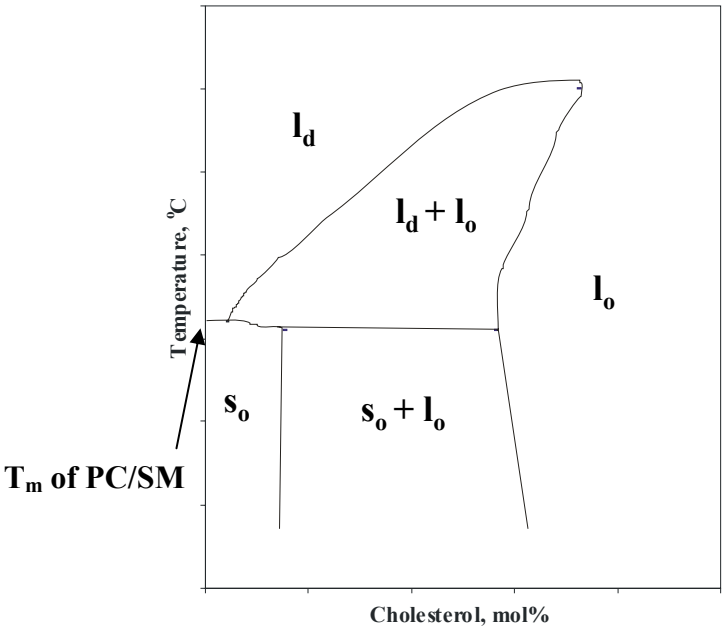

***Figure 2. A schematic phase diagram of a binary mixture of a saturated PC /SM and cholesterol as adapted from** [8, 34, 35, 96, 99, 123].*

Separation into l_o and l_d phases occurs also in ternary mixtures containing a high-T_m lipid (a saturated PC or a sphingolipid), a low-T_m lipid and cholesterol [9, 113, 126-131]. In the ternary bilayers the effect of cholesterol depends on its concentration as very high concentrations (>50%) may suppress l_o-l_d phase separation instead of promoting it [126]. If the ternary bilayer contains a large amount of sphingolipids, cholesterol may suppress domain formation even at lower concentrations [132].

Certain lipids have a higher affinity for cholesterol-rich l_o phase than others. Phospholipids with saturated and long acyl chains partition into l_o phase and interact preferentially with cholesterol, whereas unsaturated and short-chain phospholipids prefer the l_d phase [114, 115, 133-137]. In addition, the affinity of a saturated PC with cholesterol is maximal when their hydrophobic lengths are approximately equal [138-140]. Phospholipid headgroup also affects the strength of interaction with cholesterol, the affinity appears to decrease in the order: SM > PC > PS > PE

[98]. Importantly, sphingolipids with a variety of headgroup structures have been shown to be enriched in l_o phase compared to other lipids [115, 134]. Sphingomyelin, in particular, may associate tightly with cholesterol as has been proposed in various studies [70, 98]. This interaction has been attributed to van der Waals interactions [141, 142], or hydrogen bonding between the amide group of SM and the hydroxyl group of cholesterol [8, 143, 144]. On the other hand, there are also reports suggesting the opposite, i.e. that no specific association occurs between cholesterol and SM [72-74].

Taken together, the presence of cholesterol in membranes induces lateral separation of immiscible cholesterol-rich and cholesterol-poor domains, which may be a driving force in the formation of rafts in cellular membranes.

3. DETERGENT-RESISTANT MEMBRANES (DRMs)

3.1 Lipid composition of DRMs

As pointed at above, DRMs are membrane fragments insoluble in cold, non-ionic detergents and their isolation is the most commonly used biochemical method to study domain formation in cellular membranes [3, 7, 91]. DRMs isolated from MDCK cells are enriched in cholesterol, SM and glycosphingolipids, i.e. lipids forming l_o phase, relative to the whole cell lipids [7, 145]. Enrichment of sphingolipids and cholesterol has also been shown for DRMs isolated from other cell types, such as erythrocytes [97], mast cells [146], human epidermal carcinoma cells [147] and Jurkat cells [145]. DRMs have been shown to be depleted in phospholipids, such as PE, PC and anionic phospholipids compared to the whole cell lipids [7, 145, 147].

In some cases, not all of the above mentioned lipids are necessary for DRM formation. Cholesterol may not be necessary for DRM formation in cell types that are rich in glycosphingolipids and SM, such as epithelial renal and intestinal brush border cells, because they form DRMs even upon cholesterol depletion [132, 148]. In fact, in these cells DRMs isolated both at +4 ºC and at +37 ^{o}C had essentially the same protein and lipid compositions [149] implying that sphingolipids may enhance DRM formation. Glycosphingolipids are

probably not indispensable for DRM formation and can be substituted by other lipids because it was shown that a mutant cell line deficient in glycosphingolipid synthesis had the same amount of DRMs as controls [150]. In fact, it has been suggested that glycosphingolipids may even increase the detergent solubility of model bilayers containing also SM and cholesterol, and that SM and cholesterol can be important factors contributing to detergent insolubility [151]. However, there are also contrary results, as by using a monolayer technique Nyholm and Slotte showed that Triton X-100 penetrates SM monolayers more easily than PC monolayers [152].

As expected on the basis of preferential partitioning of saturated lipids into l_o phase, in DRMs phospholipid acyl chains are more saturated than on total cellular level. This has been shown for mast cells, in which 60% of phospholipids in DRMs contained saturated or monounsaturated acyl chains whereas less than 30% of total cellular lipids had these characteristics [146]. Similarly enrichment of saturated/monounsaturated phospholipids has been observed in DRMs isolated from epidermal carcinoma cells [147] and from MDCK and Jurkat cells [145]. However, the choice of detergent was recently shown to have a significant effect on the extent of enrichment of saturated phospholipids as well as lipid classes in DRMs [145].

3.2 Relationship between DRMs and rafts

It is thought that DRMs are derived from l_o domains present in cellular membranes, and that the tight packing of lipids in this phase is responsible for the detergent insolubility [4, 10-12, 91]. Using model membranes with compositions similar to DRMs isolated from cells, Brown, London and colleagues demonstrated that detergent insolubility correlates well with the presence of l_o phase [10, 11, 113]. These authors demonstrated that the presence of l_o phase greatly enhances the formation of DRMs. In addition, it was argued that detergent does not reorganise lipids in l_d phase to create insoluble domains [10, 11, 113]. Interestingly, by using electron spin resonance techniques DRMs isolated also from cells have been proposed to be in l_o phase [153].

An uncertainty with the DRM isolation method is that the amount of insoluble lipid recovered after the detergent treatment probably is not an exact measure of the amount of l_o phase present

before the detergent addition [12]. DRM isolation can underestimate the total amount of lipid present in l_o domains before addition of detergent, as shown with model bilayers [11, 154]. It was even suggested that the low temperature used in DRM isolation may be a cause for underestimation of the amount of lipids in l_o phase in model bilayers [151]. On the other hand, DRM isolation has been suggested also to lead to an overestimation of the amount of raft lipid because low temperature increases the total amount of lipid in l_o domains [12]. Besides the low temperature, the addition of detergent itself could also grow the size and number of ordered domains [155]. Detergent was suggested to even create ordered domains in an initially homogeneous, disordered PC-SM-cholesterol model membrane, and these domains are, in turn, detergent resistant upon subsequent membrane solubilisation [155].

In addition to conveying an inaccurate view on the total amount of lipid in l_o domains, detergent may also cause rearrangements of lipids [12]. After detergent treatment rafts in the plasma membrane can redistribute into a significantly more clustered distribution [156]. Interestingly, regardless of the lipid composition of the bilayer, a DRM residue consisting of PC/SM/cholesterol 1:1:1 was recovered from model bilayers in one study [151]. The DRM method can also specifically distort the lipid composition of membrane domains. In model membranes it was shown that DRM isolation can underestimate the raft affinity of lipids with short, saturated or monounsaturated acyl chains, because they have a significant affinity for l_o phase but not for DRMs [114]. Also a comparison of raft isolation by a method based on density gradient centrifugation without detergent and the DRM method revealed significant differences in the lipid composition of rafts isolated by the different methods [147]. Isolation of rafts as DRMs leads to enrichment of cholesterol, which accounted for 60% of lipid in DRMs and 25% in non-detergent lipid rafts. Also the concentration of saturated and monounsaturated phospholipids was high in DRMs (60%) compared to non-detergent rafts (40%). The concentrations of anionic phospholipids PS, PA and PI were underestimated in DRMs, as they corresponded to 5 % of total lipids in DRMs compared to 15% in non-detergent rafts [147].

In conclusion, isolation of membrane microdomains as DRMs may lead to ambiguous results. Even if a lipid or a protein is recovered in DRMs it may not necessarily be present in

microdomains in cellular membranes. Therefore, results obtained with the DRM method should be considered with caution.

4. SPHINGOLIPID AND CHOLESTEROL-RICH DOMAINS IN CELLULAR MEMBRANES

4.1 Plasma membrane

Among the cellular membranes the plasma membrane is the one most likely to contain rafts because it is rich in l_o-phase forming lipids, i.e. sphingolipids, cholesterol [50-55] and saturated phospholipid species [48, 147, 157], and it has been shown to contain substantial amounts of Triton X-100 insoluble membranes [156, 158]. The postulated interaction between SM and cholesterol may play a role in formation of plasma membrane domains because a number of studies indicate that changes in plasma membrane SM level affect the cholesterol balance of the cell [159-163]. It was first shown that depletion of plasma membrane SM by exogenous sphingomyelinase (SMase) leads to cholesterol translocation to intracellular membranes [160, 161]. Subsequently, it was suggested that only a minor amount of cholesterol moves to intracellular membranes, whereas most cholesterol remains on plasma membrane upon SM degradation [164]. The plasma membrane cholesterol may redistribute so that it is more readily effluxed to extracellular acceptors, as was shown both after SM degradation by SMase [165, 166] and in mutant cells deficient in SM synthesis [167]. In fact, cholesterol redistribution was suggested to involve cholesterol movement out of rafts [167]. However, the exact effect of SM depletion on cellular cholesterol location remains unknown.

Visualisation of plasma membrane microdomains in living cells is complicated, because the size of rafts is below the resolution of the light-microscope. Patching of raft markers by antibodies leads to appearance of visible domains on the plasma membrane [168, 169]. Patching does not, however, prove the existence of interactions of raft components in the absence of this perturbation. Evidence for plasma membrane domains in unperturbed cells has been obtained by a variety of techniques and have led to raft size estimates varying between ~26-700 nm [170-175], or even as small as 15 GPI-anchored protein molecules (~4 nm) [176]. In unstimulated

cells rafts could be small and dynamic, but may cluster into larger, more stable structures upon ligand binding to GPI-anchored or transmembrane receptors [24]. The involvement of the cytoskeleton in domain formation in the plasma membrane has also been suggested [177-179]. Electron spin resonance studies have indicated that isolated plasma membrane vesicles contain large amounts of l_o phase [180, 181], and that raft-like domains may exist in the envelope of influenza virus [182].

Due to the transbilayer asymmetry, the raft-forming sphingolipids are mostly located in the outer leaflet of plasma membrane (**Fig. 1B**). There is evidence suggesting, however, that an ordered lipid environment exists also in the inner leaflet and that the ordered domains in the two leaflets are coupled. First, Src-family tyrosine kinases in the inner leaflet associate with rafts only if their acyl groups have an extended, ordered structure as shown by their DRM association and fluorescence microscopy [15, 16]. Second, coupling of outer leaflet receptors and their inner leaflet effectors has been shown microscopically in the plasma membrane during signaling events [16, 25, 168, 183, 184]. Third, isolated DRMs have a bilayer appearance [7] and in model membranes l_o phase extends through both monolayers [127-130]. Fourth, PS and PE of the plasma membrane are enriched in saturated acyl chains [146, 157] (***II***). Notably, however, lipid rafts isolated with a detergent-free method were enriched in PE alkenyl species containing arachidonic acid, which may indicate that these phospholipids represent a significant portion of cytosolic leaflets of rafts [147]. Fifth, the long sphingolipid acyl chains present in outer leaflet may interdigitate into the inner leaflet and thus help to stabilise the domains there [3, 185]. In contrast, however, a model membrane study indicated that lipid mixtures mimicking composition of inner leaflet of plasma membrane failed to form l_o domains [186].

4.1.1 Caveolae

Plasma membrane contains special flask-shaped invaginations known as caveolae, which are present on many types of cells [187, 188]. Caveolae are characterised by their association with a family of cholesterol-binding proteins called caveolins, which are integral membrane proteins essential for the formation and stability of caveolae [189, 190]. Several GPI-anchored proteins, membrane receptors, transporters and lipid-modified cytoplasmic tyrosine kinases and

heterotrimeric G-proteins have been reported to be localised in caveolae [190-192]. As a result, caveolae mediate diverse cellular functions, such as cell signaling, transcytosis, endocytosis and exocytosis [190, 193]. Caveolae are enriched in the same lipids as rafts, they require cholesterol for their function and are also detergent-insoluble [190, 192, 194]. Therefore, caveolae can be defined as caveolin-containing plasma membrane microdomains rich in the same lipids as rafts [190, 193]. Plasma membrane microdomains are, however, by no means restricted to caveolae, as DRMs are abundant also in cells that lack caveolae [195, 196]. Thus, caveolae and rafts could be viewed as separate, but related microdomains.

4.1.2 Functions of plasma membrane domains

Plasma membrane microdomains, i.e. rafts and caveolae, have been proposed to be involved in signaling in many types of cells, especially in hematopoietic cells [16, 25, 168, 183, 184]. Antigen binding triggers cross-linking of GPI-anchored or transmembrane receptors on the exoplasmic leaflet of plasma membrane, which causes small microdomains to coalesce to form larger and more stable rafts. These larger domains facilitate interaction between aggregated receptors and Src-family tyrosine kinases of the inner leaflet, which in turn induces downstream signaling. Hydrolysis of raft SM into ceramide by cellular SMase has been implicated in the coalescence of microdomains into larger platforms enabling signal transduction in response to select agonists such as apoptotic signals or pathogens [26, 197]. In addition to signaling, cell migration in response to chemoattractants may require asymmetric domain segregation on plasma membrane to induce chemokine receptor activation [30].

4.2 Secretory membranes

Rafts may be involved in protein and lipid sorting in the Golgi apparatus. It has been proposed that in *trans*-Golgi network (TGN) glycosphingolipids form detergent-insoluble microdomains which sort newly synthesised sphingolipids and apical proteins into transport vesicles targeted to apical plasma membrane in MDCK cells [7, 17, 57, 198]. Also in unpolarised cells, sorting of newly synthesised raft proteins into distinct transport vesicles occurs in TGN (**Fig. 3**) [3, 199, 200]. Transmembrane proteins may be sorted in the Golgi based on preferential interactions

between lipids and proteins with the best matching hydrophobic length. The cholesterol and sphingolipid-gradient through Golgi is expected to gradually increase the thickness of the Golgi membranes [55] because l_o domains are likely to be thicker than a membrane in l_d phase [201, 202]. Transmembrane domains of Golgi-resident proteins are shorter than those of plasma membrane proteins, therefore, Golgi proteins may be excluded from the thicker microdomains destined for the cell surface, whereas plasma membrane proteins would be included in such domains [203].

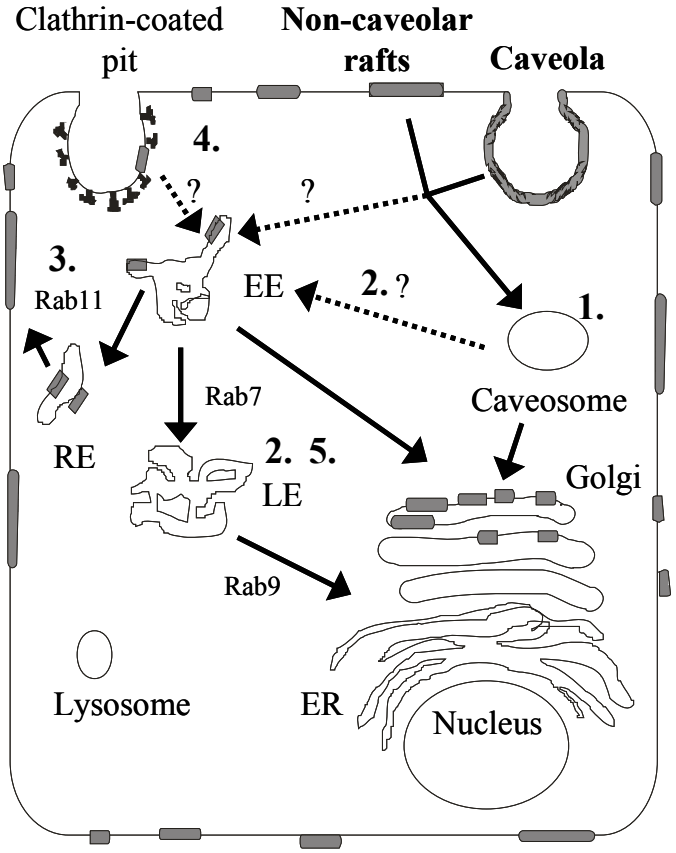

Figure 3. Possible locations of rafts in mammalian cells and routes for ligands internalised via caveolar or raft-mediated endocytosis.
Ligands internalised via caveolae or rafts have been reported to:
1. traffic to Golgi or ER via caveosomes [193, 204, 205].
2. traffic to endosomes by an unknown mechanism and from endosomes to Golgi [206, 207].
3. recycle to plasma membrane [63, 208-211].
4. be internalised via clathrin-coated pits [206, 212-219].
5. be transported both from early and late endosomes to Golgi via a mechanism dependent on raft lipid levels [207, 216-218, 220-224].

Abbreviations: EE = early endosome, LE = late endosome, RE = recycling endosome, Rab = a small GTPase functioning in vesicle docking and fusion events.

4.3 Endocytic membranes

There are several types of endocytosis in mammalian cells. The best-studied one is internalisation via clathrin-coated pits which pinch off to form clathrin-coated vesicles. Clathrin-coated vesicles fuse with early endosomes, from where the internalised components can either be recycled back to the plasma membrane or sorted to late endosomes and lysosomes for degradation. Early endosomes contain tubular elements involved in recycling and multivesicular elements directed towards endosomal degradation pathway [22]. To separate the circuits of recycling and degradation, selective sorting of lipids and proteins takes place in early endosomes [20, 22]. Sorting takes place also in late endosomes which contain large amounts of a lipid called lysobisphosphatidic acid (LBPA) in their internal membranes. These internal membranes have been suggested to be important in transport and sorting of proteins and lipids through late endosomes [64, 223]. It is thought that rafts have an important function in these sorting events within endocytic organelles [21, 22].

4.3.1 Endocytosis via caveolae and rafts

Microdomains have been implicated at the early stages of endocytosis at the plasma membrane, and this type of endocytosis is often divided into caveolar and raft-mediated uptake (**Fig. 3**). Caveolae seem to be involved in the endocytosis of various molecules, such as GPI-anchored proteins, interleukin-2 receptor and other receptors as well as their ligands [190, 193, 205, 225]. In addition, some markers experimentally introduced into cells, e.g. simian virus 40 (SV40) [204], cholera toxin subunit B (CTxB) [205, 206] and fluorescent BODIPY-labeled glycosphingolipid analogues [206, 219] have been shown to be endocytosed via caveolae. Internalisation via caveolae, however, is probably a signal-mediated process [204, 226] because in unstimulated cells caveolae appear to be largely stable and immobile structures stabilised by the actin cytoskeleton [227, 228] and caveolin-1 [229]. Caveolae, however, do not constitute the only lipid domain-dependent form of endocytosis because uptake of some raft-associated components, such as of CTxB [230, 231], GPI-anchored proteins [210] and interleukin-2 receptor [196] has been reported to take place also independently of caveolin.

It is not clear whether caveolar/raft-mediated endocytosis overlaps with the classical clathrin-mediated endocytosis (**Fig. 3**). Internalisation of some ligands via caveolar/raft-mediated endocytosis has been reported to occur independently of clathrin-coated pits [191, 193, 196, 204-206, 210, 214, 219, 231], and of these markers, SV40 [193, 204], CTxB and some GPI-anchored proteins [205] can be transported directly to the Golgi or ER via an intracellular caveolin-1-containing compartment termed as caveosome, thus passing entirely the classical endosomal circuit. On the other hand, markers can be internalised from the cell surface also both by caveolar/raft-mediated and clathrin-dependent pathways [206, 212-219], which is also supported by data showing that removal of cholesterol from plasma membrane inhibits both caveolar/raft-mediated endocytosis [206, 214, 230] as well as endocytosis through clathrin-coated pits [232, 233].

4.3.2 Domains in endocytic organelles

Rafts appear to be involved in lipid and protein sorting in early endosomes [21, 22]. Several studies suggest that rafts in early endosomes are targeted towards recycling endosomes (**Fig. 3**). First, recycling endosomes are rich and late endosomes poor in raft lipids [62, 65]. Second, the GPI-anchored folate receptor, a raft-associated protein at the plasma membrane, followed the same route as rafts into recycling endosomes where it was retained in a cholesterol- and SM-dependent manner [208-210]. Third, Pagano and coworkers recently observed BODIPY-LacCer microdomain formation in early endosomes, and suggested that BODIPY-LacCer-rich domains recycled to plasma membrane, whereas BODIPY-LacCer-poor domains were transported via late endosomes to Golgi [211]. Fourth, low density lipoprotein (LDL)-derived cholesterol was not DRM-associated after cholesterol ester hydrolysis, which also suggests depletion of rafts from late endosomes/lysosomes [234].

Notably, there are also data indicating that rafts may be directed towards late endosomal membranes. First, fluorescent lipid analogues partitioning into fluid phase recycle to cell surface, whereas lipid analogues partitioning into ordered phase traffic towards late endosomes/lysosomes (*see section 5.2*). In agreement, internalised natural glycosphingolipids, are targeted mostly to degradative membranes [235]. Second, rafts are likely to be involved in

trafficking from endosomes to Golgi (**Fig. 3**). This is suggested by the fact that natural glycosphingolipids can be sorted from endosomes to the Golgi instead of lysosomal degradation [235, 236]. In addition, several markers, such as CTxB [207, 216, 217, 222], Shiga toxin subunit B [218, 220, 221] and ricin [218], which are toxins binding to glycosphingolipids on cell surface, as well as BODIPY-LacCer [207], proteins, cholesterol [223] and different glycosphingolipids [224] are transported from endosomal membranes to the Golgi by pathways dependent on raft lipid levels.

4.3.3 Late endosomal/lysosomal lipid storage disorders

One way to study rafts is by using cells in which the balance of raft lipids in endosomal membranes is impaired. In a variety of neurodegenerative lysosomal storage diseases lipids accumulate in late endosomes or lysosomes [23, 237]. In Niemann-Pick C (NPC) disease LDL-derived cholesterol accumulates in late endosomes or lysosomes due to a defect in NPC protein which is involved in intracellular cholesterol trafficking [23, 224, 238, 239]. The disease is caused by mutations in either of the two identified genes, NPC1 or NPC2 [240, 241]. In NPC the hydrolysis of LDL cholesteryl esters is normal [242], but there is a delay in cholesterol transport to cell surface and ER [243] which may be linked to abnormal cholesterol trafficking via Golgi [165, 244]. Therefore, in NPC cells cellular cholesterol levels rise and the homeostatic responses that normally keep cellular cholesterol levels within a tight range are defective [238, 242]. In addition to cholesterol, sphingolipids such as ganglioside GM_2 [245], GM_1 [206, 207] and other glycosphingolipids [224] accumulate in NPC cells.

In sphingolipid storage diseases (SLSDs) different sphingolipids accumulate in late endosomes/lysosomes. In most SLSDs the sphingolipid build-up results from a deficiency in lysosomal sphingolipid hydrolysis. Similarly to sphingolipid build-up as a consequence of cholesterol accumulation in NPC, cholesterol becomes deposited into endocytic organelles in SLSDs [246] as well as in normal cells loaded with sphingolipids [247].

Pagano and coworkers have observed that in SLSDs (including NPC) trafficking of exogenous BODIPY-LacCer is different from normal cells [246, 248, 249]. Similar results were obtained with BODIPY-labeled SM, GalCer, GM_1, globoside and sulfatide [237, 250]. Normally

BODIPY-LacCer becomes enriched in the Golgi, whereas in SLSDs it becomes trapped in punctate endosomal structures of which only 30-40%, however, correspond to late endosomes/lysosomes [237, 246]. This is compatible with results suggesting that CTxB bound to GM_1 accumulates in early endosomes in NPC [222] and that sphingolipid and cholesterol were shown not to deposit in lysosomes [247]. Taken together, the studies on SLSD cells indicate that an increase in one raft lipid causes an increase in other raft lipids in endosomal membranes, which implies that microdomains are involved in endosomal trafficking (*see section 4.3.2*). Indeed, association of cholesterol with DRMs is increased in NPC as compared to normal cells [234, 251]. It has been proposed that in SLSD cells rafts accumulate in late endosomes and this leads to a traffic jam [21, 23].

In conclusion, numerous studies support the view that cell membranes rich in sphingolipids and cholesterol, i.e. the plasma membrane, the Golgi and some endosomal membranes, may contain functional microdomains. To study the microdomains is difficult because they cannot be isolated in their native state, and are presumably too small and transient to be directly observed under the microscope. Thus, much of the data on the domains is based on indirect methods. Many questions still remain open, for example, the size, shape and lifetime of microdomains, and the relationships between rafts and caveolae.

5. FLUORESCENT LIPID ANALOGUES AS TOOLS TO STUDY MEMBRANE DOMAINS AND LIPID SORTING

5.1 Fluorescent lipids commonly used to study trafficking and domains

5.1.1 General properties

To date most experimental evidence for lipid sorting based on differential partitioning into ordered domains has largely been derived from studies examining the intracellular trafficking of short chain, NBD- or BODIPY-labeled sphingolipids. The advantage of these lipid analogues is that due to their polarity they readily diffuse into cells when added into culture medium. The disadvantage of using NBD-labeled lipids is that because of the polarity of the NBD group the

labeled acyl chain loops back to the membrane-water interface independent of the acyl chain length [44-46]. Therefore, these analogues may not behave the same way as natural lipids. BODIPY-labeled lipids are attractive for trafficking studies because the BODIPY moiety exhibits a shift in its fluorescence from green to red wavelengths with increasing concentrations in membranes. This property can be employed to estimate the concentration of BODIPY-labeled lipids in cellular membranes [250, 252]. BODIPY is more hydrophobic than NBD, but it also has some tendency to move towards the membrane surface when attached to the end of an acyl chain [253].

Recent model membrane studies on partitioning of NBD- and BODIPY-labeled sphingolipids between different membrane domains, have demonstrated that these analogues do not partition significantly into l_o domains, but prefer l_d phase, which is contrary to the domain partitioning of natural sphingolipids [115, 130]. This may reflect the fact that due to looping back of the labeled acyl chain, the analogues occupy a larger molecular area than their natural counterparts preventing them from tightly interacting with ordered domains.

Lipid-mimicking DiIC$_n$ dyes, on the other hand, show preferential partitioning into gel phase as their alkyl chain length increases, to match the hydrophobic thickness of the host lipid acyl chains [254, 255]. DiIC$_n$ as well as DiOC$_n$ analogues have been used in lipid trafficking studies in cells [20]. Silvius and colleagues have used various fluorescent phospho- and sphingolipid analogues with different chain lengths to study lipid partitioning between ordered and disordered domains in model membranes. They used lipids with 1,6-diphenylhexatrienyl- and indolyl-labeled acyl chains, which position the fluorescent group deep within the bilayer interior, to study which structural characteristics target lipids into l_o domains [115, 134]. Also headgroup-labeled bimane lipids have been used in a related study, because the bimane group is uncharged, relatively small and does not seem to affect probe binding [114].

5.1.2 Cellular studies

Endocytosis of NBD-SM has been studied in various cell types. Most of NBD-SM was shown to be transported along the recycling pathway and only a small amount was sorted to the degradative pathway in CHO cells and human fibroblasts [256-258]. Sorting of NBD-SM away from the degradative circuit occurred in the early endosome [258]. Cells labeled with NBD-SM showed also staining of the Golgi within ~1.5 hours which was probably due to SMase-induced hydrolysis of NBD-SM into NBD-Cer, which can spontaneously diffuse between membranes [256, 257].

BODIPY-SM has also been reported to label many endosomal structures as well as to traffic to Golgi with longer incubation times in human fibroblasts [109, 250, 259, 260]. Also with this probe the trafficking to Golgi could be due to the hydrolysis of BODIPY-SM into BODIPY-Cer [250, 259, 260]. Puri and colleagues have proposed that the internalisation of BODIPY-SM occurred approximately equally by caveolar and clathrin-mediated pathways [206]. In SLSD cells, however, BODIPY-SM translocated to both punctate late endosomal/lysosomal structures as well as Golgi [206, 250, 260].

Trafficking routes followed by fluorescent glycosphingolipids resemble to a certain extent those followed by the SM analogues. NBD-GlcCer was shown to recycle to the cell surface to a major extent, but also to traffic in 30 minutes to late endosomes and the Golgi without any breakdown into ceramide [236, 258, 261, 262]. Similar results were obtained with BODIPY-labeled GlcCer and GalCer which were in 15-30 minutes sorted mainly to the Golgi in human fibroblasts, possibly by translocation across plasma membrane and spontaneous diffusion from plasma membrane to the Golgi [259]. BODIPY-LacCer, on the other hand, was found in addition to Golgi to be localised in punctate structures in 15-30 minutes [259]. With time, BODIPY-LacCer also became enriched in the Golgi, which appeared to be as an intact molecule because after 2 hours only 7% of it was degraded [248]. Approximately one half of BODIPY-LacCer was transported to the Golgi through early and late endosomes Rab7- and Rab9-dependently [206, 207] and the other half recycled back to plasma membrane [211]. In SLSD cells BODIPY-LacCer and other BODIPY-labeled glycosphingolipids accumulate in punctate endosomal

structures instead of Golgi targeting (*see section 4.3.3*). Uptake of BODIPY-labeled LacCer, globoside, sulfatide and GM_1 from plasma membrane seems to occur via caveolar endocytosis [206, 219].

Domain-based sorting of lipids in early endosomes has been studied by lipid-mimicking $DiIC_n$ and $DiOC_n$ analogues [20, 158]. Short-chain saturated and unsaturated analogues, which partition into fluid phase, exited early endosomes rapidly for recycling to plasma membrane, whereas long-chain $DiIC_{16}$ and $DiOC_{16}$, which partition preferentially into ordered phases, were retained and consequently trafficked to late endosomes/lysosomes [20]. $DiIC_{16}$ was also shown to be in DRMs in cells [158]. BODIPY-PC with C_5-chain also recycled and BODIPY-PC with a C_{12}-chain was directed to degradative compartments, even though the difference between them was not as clear as with the $DiIC_n$ and $DiOC_n$ analogues [20].

5.2 Pyrene-labeled lipids

5.2.1 General properties

Pyrene-labeled lipids represent another type of fluorescent lipid analogues that have been used to study domain partitioning and lipid trafficking (**Fig. 4A**). The pyrene fluorophore is usually attached to the distal end of the acyl chain, and these probes are referred to as mono- or dipyrene lipids depending on whether only one or both acyl chains are labeled. Pyrene has two unique spectroscopic properties as compared to the other commonly used lipid fluorophores: it has a very long excited-state lifetime (> 100 ns) and it can form excited state dimers, i.e. excimers. At low concentrations pyrene lipids emit only monomer fluorescence, but as the concentration increases the probability of collisions between excited-state and ground-state pyrene monomers increases, which leads to formation of excimers (**Fig. 4B**). The rate of excimer formation is proportional to the *local* pyrene lipid concentration which allows one to study lateral organisation of membranes by recording the ratio of excimer to monomer fluorescence intensities (E/M) [263-265]. Partitioning of pyrene lipids between gel-state and fluid-state domains has been investigated by recording the E/M ratio as a function of temperature in model membranes [104, 266-271].

Another important advantage of pyrene lipids is that the pyrene moiety is hydrophobic, and therefore, does not strongly modify the hydrophobic character of the linked acyl chain [265]. For instance, monolayer studies have shown that pyrene does not significantly increase the surface area of a phospholipid molecule [104]. Therefore, it has been shown that the pyrene moiety attached to an acyl chain does not distort the conformation of the labeled acyl chain, but the depth of the pyrene moiety in a bilayer is fully determined by the length of the linked acyl chain [272] in contrast to NBD and BODIPY fluorophores [44, 253]. However, even a hydrophobic moiety embedded into the membrane may perturb membrane packing as shown by computer simulation studies on the effect of benzene and 1,6-diphenylhexatriene on membranes [273, 274]. On the other hand, these probes caused no significant changes in the membrane thickness or surface area per matrix lipid.

The local concentration-dependent excimer formation of pyrene lipids enables their monitoring by E/M imaging. The first study in E/M imaging reported differences between different organelles of fibroblasts incubated with a pyrene fatty acid, even though the physiological significance of this finding remained unclear due to difficulties in interpreting the data [275]. In another study changes in E/M of Pyr_4GM_1 in the plasma membrane of cultured neurons were observed and considered to reflect domains on the cell surface [276]. There are several issues, however, that need to be taken into consideration in using E/M-imaging and imaging of pyrene lipids in general [265]. First, due to its long excited-state lifetime pyrene is quenched significantly by molecular oxygen [266]. Second, photobleaching is a major problem in quantitative fluorescence imaging. Both of these factors can be eliminated by enzymatically depleting oxygen from the medium, a process which was shown not to harm the cells during the time course studied (> 20 min) [277]. Third, as many of the endosomal vesicles lie very close to the plasma membrane, visualisation of plasma membrane images may be difficult. This problem can be overcome in living cells by selective quenching of the plasma membrane fluorescence by trinitrophenyl-lysoPE (TNP-LPE) followed by image subtraction, which provides plasma membrane images free of endomembrane contribution [277]. Fourth, the background due to native cellular fluorophores can significantly contribute to the images, but it can be reasonably corrected [277].

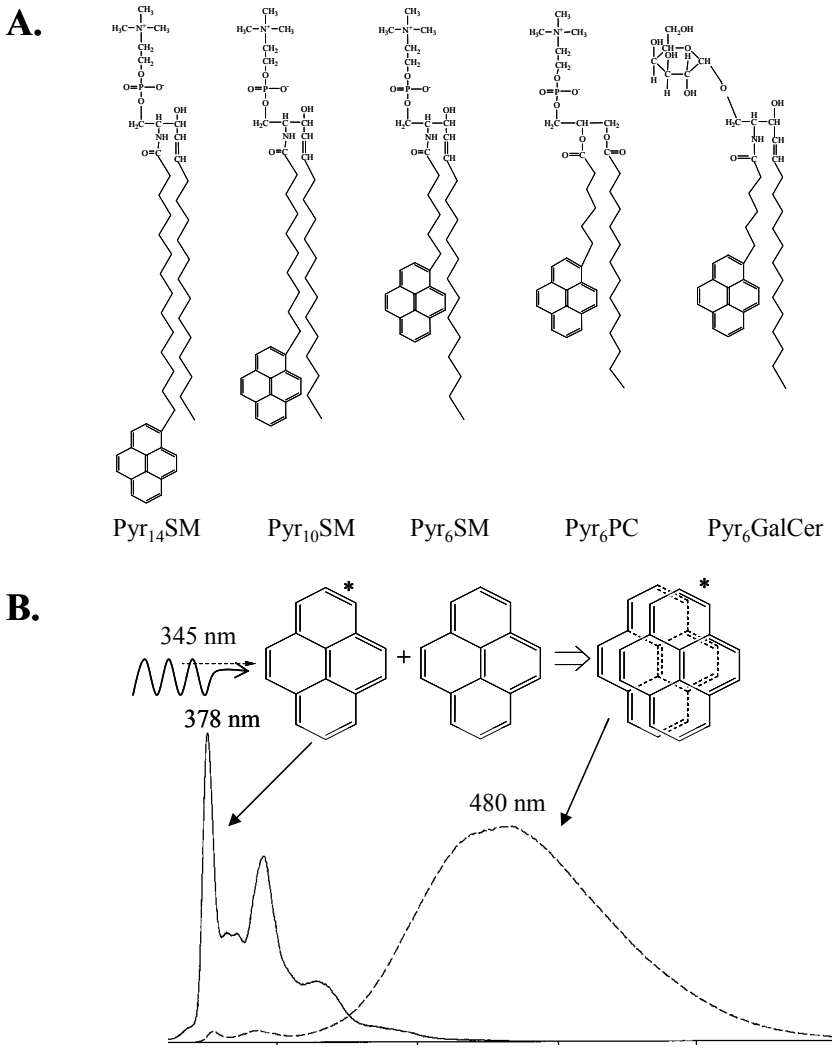

Figure 4. (A) Structures of some monopyrene lipids. (B) Normalised emission spectra for a monopyrene lipid at a low (solid line) and a high concentration (dashed line) in a bilayer.

5.2.2 Cellular studies

There are data indicating that pyrene lipids are appropriate mimics for natural lipids. Pyr$_n$ fatty acids are efficiently incorporated into phospholipids and neutral lipids in baby hamster kidney (BHK) cells similarly to the corresponding natural species [278]. The fatty acyl chain length, however, had a significant effect on the incorporation, as short-chain fatty acids incorporated much more slowly into cellular lipids than long-chain ones. The short-chain fatty acids incorporated mainly into *sn*-2 position and long-chain ones into *sn*-1 position, suggesting that

they mimic unsaturated and saturated fatty acids, respectively. Among phospholipids PC and PE species became most labeled. Also the metabolism of different diPyr$_4$ phospholipids resembled that of their natural counterparts. For instance, diPyr$_4$PA was converted into diacylglycerol, triacylglycerol and PC, diPyr$_4$PS was decarboxylated into PE, and diPyr$_4$PC and diPyr$_4$PE were not metabolised further [279]. Pyrene-labeled SM and several glycosphingolipids are hydrolysed into corresponding ceramides [280-282]. Pyrene-labeled lipids are also good substrates for phospholipid carrier proteins [283, 284].

So far, pyrene lipids have found only limited use in studies on intracellular trafficking of lipids [285, 286], largely because pyrene lipids are quite hydrophobic, and therefore, do not easily diffuse into cells when added in vesicles into the culture medium. This problem can be resolved by using pyrene lipids with rather short acyl chains, such as diPyr$_4$-derivatives, which are polar enough to spontaneously diffuse into cells [279]. However, the short acyl chains allow lipid analogues to spontaneously translocate between membranes [264, 287] which may complicate the interpretation of the data. It was recently demonstrated, however, that even the more hydrophobic pyrene-labeled lipids can be transferred from vesicles into cells by using γ-cyclodextrin (γ-CD) [285, 286]. This method is independent of cell type or structural details of the lipid. Nevertheless, even this method fails to transfer the most hydrophobic Pyr$_n$ (n >12) species into cells. Notably, pyrene lipids can be introduced into cells also via endocytosis by incorporating them into lipoproteins, even though this method targets the lipids into lysosomes [280, 281].

γ-CD mediated transfer of pyrene lipids has been used for lipid trafficking studies. It has been demonstrated that short-chain diPyr$_n$PS translocated into mitochondria and other intracellular sites and more hydrophobic long-chain diPyr$_n$PS remained largely on plasma membrane [285, 286]. Intracellular trafficking of diPyr$_4$PC has been studied in cells from patients with the lysosomal storage disorder mucolipidosis-IV. The results showed that in contrast to fluorescent sphingolipids [246], lysosomal accumulation of diPyr$_4$PC in ML-IV cells was not due to increased cholesterol content of late endosomes/lysosomes (*Jansen et al. 2003, personal communication*).

Taken together, hydrophobic, e.g. pyrene-labeled, fluorescent lipid analogues have definite advantages over their more hydrophilic counterparts. As it is now possible to introduce even the more hydrophobic pyrene-labeled lipids into cells, they are useful tools in lipid trafficking studies.

6. ELECTROSPRAY IONISATION MASS SPECTROMETRY (ESI-MS) AS A TOOL TO STUDY (PHOSPHO)LIPID COMPOSITIONS OF MEMBRANES

6.1 Advantages of lipid mass spectrometry over conventional methods

Traditional analysis of phospholipid compositions of biological samples is a complex process including possibly several chromatographic and enzymatic steps [38, 288, 289]. Typically, the analysis includes *i*) separation of glycerophospholipid classes by HPLC or TLC, *ii*) phospholipase C treatment of each class, *iii*) derivatisation of the resulting diacylglycerols, *iv*) separation of ether lipid species from diacyl species and *v*) analysis of the individual molecular species by reversed phase HPLC and gas chromatography. The methodology is also insensitive as hundreds of nanomoles of total phospholipid are needed for a complete analysis of the molecular species profiles.

Mass spectrometry (MS) offers a highly sensitive and specific alternative method for lipid compositional analysis. Initial phospholipid analyses by MS were conducted using fast-atom bombardment (FAB) as the ionisation method. However, the high-energy ionisation causes extensive fragmentation of the lipids which makes quantitative compositional analysis by FAB nearly impossible [39, 290, 291]. Electrospray ionisation (ESI) is a soft ionisation method, and therefore, it is a very promising technique in lipid compositional analysis [42]. ESI-MS enables the quantification of hundreds of different lipids including those of low abundance and it can resolve more molecular species than HPLC [39, 292]. Its sensitivity allows analysis from picomole amounts of lipids [39, 40, 293, 294]. In addition, the instrument response has been shown to be linear over at least 2 orders of magnitude in the femtomole-picomole concentration range [39, 295].

Matrix-assisted laser desorption (MALDI) ionisation has also been used in the analysis of phospholipids. However, the presence of the peaks arising from the matrix impair the sensitivity, particularly in negative ion mode [296]. An even more serious drawback is that PC strongly suppresses ionisation of the other phospholipids both in the positive and negative ion mode, which may cause problems for detection of phospholipids in crude extracts of biological material without prior separation into phospholipid classes [296, 297].

6.2 Quantification of phospholipids by ESI-MS

The unique advantage of ESI-MS is that it allows rapid quantification of phospholipid molecular species directly from crude lipid extracts, i.e. without prior chromatographic separation of phospholipid classes [39, 40, 42, 292]. The zwitterionic PC and SM can be analysed as positive molecular ions $[M+H]^+$ and the anionic phospholipids, i.e. PS, PI, PA, as well as PG and PE as negative molecular ions $[M-H]^-$. Alternatively, PC, SM and PE can be analysed as their Na^+-adducts in positive ion mode [39, 292, 293]. Also LiOH can be added into samples to convert PC and SM into positive Li^+-adducts and simultaneously to increase the signal of PE in negative ion mode due to deprotonation of PE molecules [41, 42, 298-300]. Very importantly, the different lipid classes can be analysed selectively, i.e. without interference by other classes, by using class specific scanning modes. For instance PC and SM can be analysed by scanning for precursor ions for a fragment of *m/z* 184, PE by scanning for a neutral loss of 141 and PS for a neutral loss of 185 in the positive ion mode [40, 157, 301].

Molecular species from crude extracts can be identified by MS/MS. For instance, anionic phospholipid species can be identified by negative-ion MS/MS to detect the acyl carboxylate ions [41, 292, 293] or by precursor ion scanning for all potential acyl carboxylate ions [41, 42]. PC and SM molecular species have been characterised by converting them into Li^+-adducts and daughter ion scanning or precursor ion/neutral loss scanning in positive ion mode for fragments [41, 298, 299]. Similarly, PE species can also be structurally characterised as Li^+-adducts [302].

Internal standards consisting of molecular species not occurring naturally are often added into samples to accurately quantify lipid compositions. According to some reports, phospholipid acyl

chain length or unsaturation has no affect on the ionisation efficiency in MS mode at subpicomolar lipid concentrations [39]. Therefore, addition of only one internal standard for each lipid class would be adequate when measuring very diluted samples [42, 147, 292]. However, in the MS/MS mode differential fragmentation rates of the individual molecular species have been demonstrated [40, 303] indicating that a set of internal standards consisting of different molecular species for each phospholipid class should be included to obtain accurate quantification [40].

AIMS OF THE PRESENT STUDY

The main goal of this study was to develop and apply novel methodologies, electrospray ionisation mass spectrometry (ESI-MS) and domain partitioning of pyrene-labeled lipids to obtain information on lipid composition and properties of domains in model and cellular membranes. The more specific aims were:

1) To examine various factors affecting quantitative analysis of lipid compositions by ESI-MS.

2) To use ESI-MS to determine the lipid composition of plasma membrane domains (ordered vs. disordered), and the effect of chronic and acute cholesterol load on phospholipid compositions of these domains as well as those of whole cells.

3) To characterise partitioning of fluorescent, pyrene-labeled phospho- and sphingolipids between ordered and disordered domains in model membranes to allow their use in studying domain organisation and raft lipid trafficking in living cells.

EXPERIMENTAL PROCEDURES

All the experimental procedures used in the original articles and unpublished data are summarised in the table below. The number of the original publication in which the method has been described is indicated.

METHOD	
Cell culture of BHK-21 cells	*I*
Cell culture of human fibroblasts	*II*
Cholesterol loading of fibroblasts	*II*
Differential Scanning Calorimetry	*III*
DRM isolation DRMs were isolated by flotation at +4 °C in an Optiprep gradient in the presence of 1% Triton X-100 as previously described [168].	
Electron microscopy	*II*
Fluorescence imaging Fibroblasts were imaged on glass bottom dishes (MatTek Corporation, Ashland, USA) in CO_2-independent minimum essential medium (I-MEM) containing oxygen-depletion reagents as previously described [277, 285]. To selectively quench fluorescence of the plasma membrane, 1ml of TNP-LPE solution in PBS (30 mM) was added to the dish containing 2 ml of I-MEM. Imaging was carried out on TILLPhotonics imaging system (Gräfelfing, Germany) equipped with a flexible excitation light source and an Olympus IX70 (Melville, USA) cooled CCD camera driven by TILLvision 4.0 software (Gräfelfing, Germany). For pyrene lipids 405 nm (40 nm bandpass) and 480 nm (60 nm bandpass) emission filters were used.	
Fluorometry	*III*
Glycosphingolipid analysis	*II*
HPLC analysis of Pyr$_n$SM metabolism Lipid extracts were analysed as previously described [304]. On-line fluorescence detection was used to calculate the amounts of Pyr$_n$SM and their metabolic products.	
Introduction of Pyr$_n$SM species to the plasma membrane of human fibroblasts γ-CD was used to transfer pyrene labeled sphingomyelins to the plasma membranes of	

fibroblasts [285]. Pyr$_n$SM in 100 mM γ-CD in PBS was probe sonicated for 3 x 2 minutes at room temperature to form Pyr$_n$SM/γ-CD complexes. 80-90% confluent fibroblasts monolayers were labeled with the complex for 5 minutes at +37 °C in serum-free Eagle's minimum essential medium (E-MEM). The final lipid concentration was adjusted to 6.7 μM and γ-CD concentration to 6.7 mM. For Pyr$_{16}$SM the cells were initially labeled at 30 mM γ-CD for 4 minutes, followed by dilution of the labeling medium to 10 mM γ-CD for 4 minutes, as this protocol gave a more efficient labeling efficiency. After the labeling cells were washed with PBS and E-MEM, and chased for the desired time in serum-free E-MEM. For fluorescence microscopy, cells were chased in I-MEM.
Labeling with ^{3}H-choline chloride Before the isolation of DRMs fibroblasts were labeled for 24 hours with ^{3}H-choline chloride at a concentration of 4 μCi/ml in serum-containing E-MEM.
Lipid extraction *I+II*
Mass spectrometry of cholesterol *II*
Mass spectrometry of phospholipids *I+II*
Multilamellar liposomes *III*
Phosphorus determination *I+III*
Protein determination *II*
Removal of detergent from cellular lipid extracts To analyse lipid composition of DRMs by ESI-MS, Triton X-100 was removed from cellular lipid extracts with a reversed phase HPLC cartridge (OASIS HLB, particle size 2.5 μm, 2.1 x 20 mm, Waters, Toronto, Canada). Lipids were trapped into the column with methanol/H_2O 2:1, and eluted out with increasing amounts of chloroform/methanol 1:1.
Synthesis of MS standards *I+II*
Synthesis of pyrene-labeled lipids *III*
Thin layer chromatography *I*
Virus purification *II*

RESULTS AND DISCUSSION

1. QUANTITATIVE DETERMINATION OF PHOSPHOLIPID COMPOSITIONS BY ESI-MS (*I*)

We studied several issues that need to be taken into account to achieve truly quantitative compositional data on phospholipids with ESI-MS. The effects of acyl chain length, unsaturation, polar headgroup structure and lipid concentration on instrument response were studied. We then tested whether the phospholipid composition of BHK cells could be determined quantitatively by ESI-MS by comparing the results with those obtained with conventional methods.

1.1 Effects of acyl chain length, unsaturation and polar headgroup on instrument response

Acyl chain length was found to have a significant effect on the instrument response. The response decreased markedly and in a nearly linear manner with increasing acyl chain length both for the saturated and diunsaturated species (***I, Fig. 1***). Although this has been shown previously for saturated species [40], the novel finding was that the lipid concentration strongly modified this chain length-dependency of instrument response. At the highest concentration studied (10 pmol/μl per species, 120 pmol total lipid/μl), the relative response decreased strongly and in a seemingly exponential manner with the acyl chain length as shown in **Fig. 5** (***I, Fig. 2***), as also reported previously [40]. However, when the concentration was decreased the response became a linear function of the chain length (**Fig. 5**, ***I Fig. 2***). Parallel results were obtained for the (di)unsaturated PC species (***I, Fig. 3*****)** as well as for PE, PS and PA standards (data not shown).

Phospholipid acyl chain unsaturation also had a marked influence on the instrument response, and this effect also depended on total lipid concentration as shown in **Fig. 6** (***I, Fig. 4***). At high concentrations, the response for the polyunsaturated species was 40 % higher than that for the fully saturated ones. However, upon dilution the effect of unsaturation gradually diminished and

virtually disappeared (at 0.1 pmol/μl per species, 1.4 pmol/μl of total lipid). Although a similar effect has been observed for triacylglycerols previously [305], ESI-MS studies on phospholipids have not indicated this phenomenon before. A possible explanation is the low concentrations used in most studies [39], therefore, effects of acyl chain unsaturation as well as chain length may have gone unnoticed.

The peak intensity was also influenced by the phospholipid headgroup structure. In the negative ion mode using chloroform/methanol (C/M) 1:2 as the solvent, acidic phospholipids PG, PI, PA and PS gave a much higher response than PE or the chloride adduct of PC (***I, Fig. 5A***). Inclusion of 1% NH_4OH in the infusion solvent increased the response for PE markedly (***I, Fig. 5B***) probably because the higher pH favors a loss of proton from NH_4^+-group of PE [42]. Also LiOH or NaOH can be used to achieve this effect [39, 41, 299, 300]. In the positive ion mode, with C/M 1:2 as the solvent, the highest peak was observed for protonated PC followed by the Na^+-adducts of other lipids (***I, Fig. 5C***). Formation of Na^+-adducts of PC has been reported before [40, 294, 295]. Na^+-adducts can complicate the analysis of the spectra. This problem was overcome in the present study by inclusion of NH_4OH, which caused the intensity of protonated PC by far to exceed that of the other lipids (***I, Fig. 5D***). This has also been noted by another study [301]. Alternatively, NaOH or LiOH has been used to force the formation of Na^+ or Li^+ adducts for PC and SM [39, 41, 298-300]. However, we were not able to fully suppress the formation of protonated molecular ions with NaOH (data not shown).

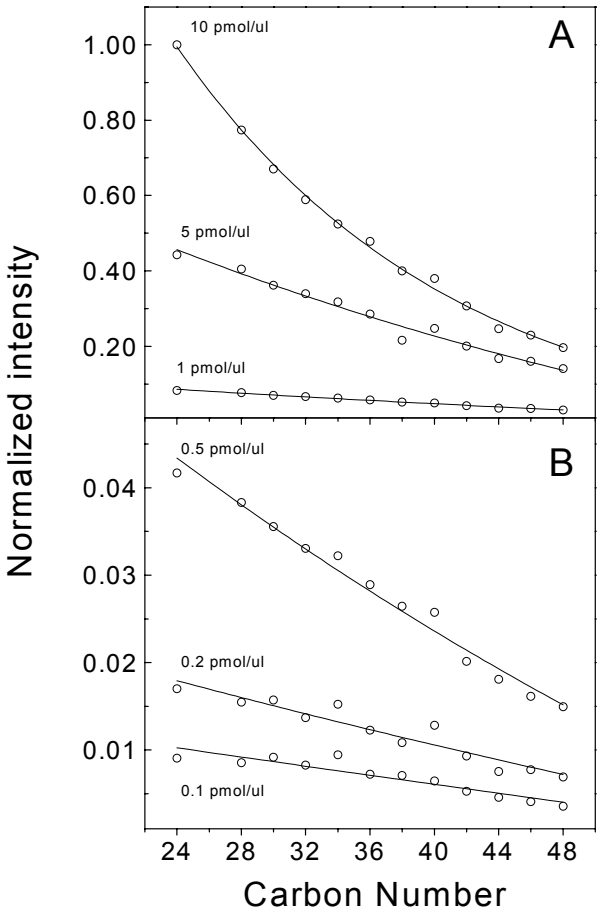

Figure 5. Effect of concentration of saturated phosphatidylcholines on instrument response. An equimolar mixture of 12 saturated PC species of the indicated chain length was prepared, diluted to obtain the indicated concentrations per species and then analysed (*see **I** for details*). *(A)* Data for the three highest concentrations, *(B)* data for the three lowest concentrations. Each data point is an average of 5 replicate samples with standard deviation of less than 5 %. The infusion solvent was C/M 1:2 + 1 % NH_4OH.

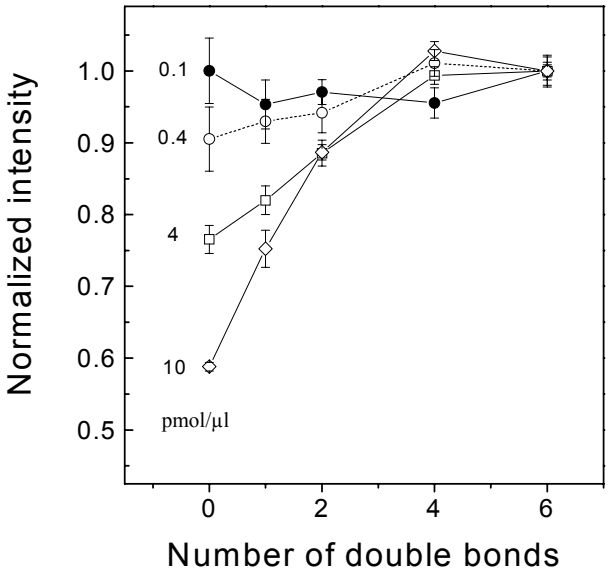

Figure 6. Effect of degree of unsaturation on instrument response at different concentrations. An equimolar mixture of 14 different PC species including five species with 36 acyl carbons and 0, 1, 2 , 4 or 6 double bonds was diluted to indicated concentrations per species (1.4 - 140 pmol/µl total lipid) and then analysed (*see **I** for details*). The response values have been normalised relative to that for the 36:6 species at each concentration. The error bars indicate the standard deviation (n = 5).

1.2 Effect of lipid concentration on instrument response

The linearity of instrument response is obviously an important concern in quantitative analysis. Our analyses revealed that the intensities were linearly dependent on the lipid concentration up to about 1 pmol/μl, but tended to level-off at higher concentrations (***I, Fig. 6***). A recent study supports our data [306]. Such a signal saturation is a typical phenomenon in ESI-MS and results from the saturation of surface of the spray droplets by the analyte molecules. Importantly, however, the responses *relative* to an internal standard were linearly dependent on the concentration up to at least 5 pmol/μl. Such linear behavior was obtained for phospholipids even in the presence of other cell-derived lipids and other compounds present in the crude lipid extract (data not shown). The relative response was a linear function of lipid concentration over a range covering two orders of magnitude, as shown also previously for mere lipid standards with a quadrupole instrument [39, 295].

1.3 Analysis of phospholipid molecular species composition of BHK cells

To determine quantitatively the molecular species profiles of the major phospholipids of BHK-21 cells, a total lipid extract was spiked with at least three internal standards for each major phospholipid class (***I, Fig. 7***) and then analysed by a triple quadrupole instrument using class specific neutral loss- or precursor ion scanning [40, 301]. The concentration of each molecular species in a phospholipid class could be calculated by using a correction function obtained by plotting the measured intensity of the internal standards vs. *m/z*. The saturated standards used allowed us to correct for the acyl chain length-dependency of instrument response, but not for the unsaturation dependency. Dilution of the samples, however, should greatly diminish the effect of acyl chain unsaturation [42], but even then it may not be eliminated, particularly if impurities are present in the sample. As a result (see below), it would be safer to employ unsaturated standards because their response is more similar to natural phospholipids [307].

The determination of the molecular species of phospholipid classes in BHK cells (***I, Table 1***) showed that the major PC species were 34:1 (25 %) and 36:2 (23 %), while the major PE species were 36:2 (25 %) and 36:1 (10%). The most abundant PI species were 38:4 (23 %) and

38:3 (19 %), while the most prominent PS species was 36:1 (31 %). The most significant SM probably corresponded to a lipid with a sphingosine backbone and a 16:0 acyl chain. [40, 55, 60] After quantification of each molecular species, the total amount of lipid in a phospholipid class was be calculated. The accuracy of phospholipid class quantification by the MS method was tested by comparing ESI-MS results with quantification by TLC and phosphorus analysis from the same extract of BHK cellular lipids. The abundances of most phospholipid classes determined by the ESI-MS method agreed very well with those determined by the TLC method here (***I, Fig. 8***) or previously [308], indicating that ESI-MS yields reliable quantitative data.

This is the first ESI-MS study in which all the major phospholipid classes have been quantified with the use of three internal standards for each species to account for the chain-length dependency of instrument response. In most other studies only a single internal standard for each class has been used, and effects of chain length or unsaturation were thought to be alleviated by diluting the samples [41, 42, 147]. Such extensive dilution, however, has obvious drawbacks. First, detection of the minor species is compromised. Second, the effects of unsaturation and chain length may not disappear even by dilution if samples contain surface-active impurities, such as detergents. Third, when using class specific detection the differential fragmentation rates of individual molecular species are a major cause of deviation in the instrument response [40, 303]. Due to these factors, inclusion of several internal standards for each phosholipid class is highly useful.

Separation of phospholipid classes by HPLC either off- or on-line before MS analysis can also be advantageous. Pre-separation causes less suppression of analytes during the ionisation, thus a linear chain length-dependency can be more easily obtained by isocratic HPLC [307]. HPLC before MS may be useful also if sample contains high amounts of detergents or other impurities interfering with the ionisation process [146, 147]. It is necessary when minor but functional phospholipids, such as PA or LBPA, are to be analysed since no specific scan modes are available for these lipids [40, 307]. Also determination of the fatty acyl residues of the individual molecular species by fragmentation analysis is more practical after separation of phospholipid classes [41, 299, 309, 310]. On the other hand, chromatographic pre-separation

may have the disadvantage of introducing artifacts due to selective loss of certain molecular species [306].

2. EFFECT OF CHOLESTEROL LOADING ON THE LIPID COMPOSITION OF HUMAN FIBROBLASTS AND THEIR PLASMA MEMBRANE DOMAINS (*II*)

In this work ESI-MS was used to quantify molecular species of the major phospholipid classes, i.e. PC, PE, SM and PS, in normal fibroblasts and in fibroblasts loaded with cholesterol either chronically (NPC) or acutely. Furthermore, lipids were quantified from enveloped viruses budding from the plasma membrane of normal and NPC cells. Influenza virus was used to isolate ordered, raft-like plasma membrane domains and vesicular stomatitis (VSV) to isolate fluid, raft-depleted domains [18, 27].

2.1 Lipid composition of control and NPC fibroblasts

In the present study, first of all, the total lipid concentrations between control and NPC cells were compared. Quantification of the fibroblast phospholipid classes showed no difference in the concentrations of glycerophospholipids or total glycosphingolipids between cellular extracts from control and NPC cells (***II, Fig. 1***). Previously in some tissues from NPC mice/humans increases in the amounts of certain sphingolipids have been observed [311-313]. The major difference between control and NPC was a ~2.5-fold enrichment of cholesterol in NPC. This is in agreement with results obtained with NPC mouse tissues and fibroblasts [311, 312, 314, 315].

Second, the molecular species composition of control and NPC cells was studied. Both in control and NPC cells the molecular species composition was significantly different between the phospholipid classes (***II, Fig. 2, Table 1***). SM had the highest content of saturated acyl species, also PC was abundant in saturates. PE, on the other hand, was the class containing the highest amount of unsaturated molecular species. These results agree well with previous reports on the degree of unsaturation of phospholipid classes in mammalian cells [316, 317]. A closer comparison of the molecular species of control and NPC cells showed, however, that the phospholipid acyl chains were modulated towards increasingly polyunsaturated species in NPC

cells. PC and PE in NPC cells contained more polyunsaturated species and less mono- and diunsaturated species compared to control cells (***II, Fig. 2)***. In accordance with these data, it has been reported that in NPC mouse tissues unsaturated fatty acids are enriched in all the major phospholipid classes [311]. Another study, however, reported that the amounts of saturated fatty acids were increased and monounsaturated fatty acids decreased in the lipids of NPC mice and fibroblasts [314]. Nevertheless, also in this study polyunsaturated fatty acids seemed to be increased. Fatty acid composition has also been reported to remain unchanged in NPC [312].

2.2 Lipid composition of fibroblasts with an acute cholesterol loading

The increased level of unsaturated fatty acids in phospholipids of NPC cells was thought to result from the cells attempting to maintain an adequate level of membrane fluidity when the cholesterol concentration of the membranes is increased. However, rather than resulting directly from the increased cholesterol content, in NPC cells the modeling of the phospholipid species towards more unsaturated ones could also be a secondary metabolic effect. For instance, β-oxidation of polyunsaturated fatty acids may be impaired due to dysfunctional peroxisomes in NPC [312, 318]. To eliminate this possibility, normal fibroblasts were loaded with cholesterol using methyl-β-cyclodextrin/cholesterol complexes as donors [319]. An efficient cholesterol loading was achieved, and in 24 h the cholesterol content of cells increased ~5-fold (***II, Fig. 7***). Such acute cholesterol loading of cells produced no changes in total SM, PS or PE levels, however, PC mass increased by ~25% (**Fig. 7**). Previously, PC biosynthesis was found to be activated and also lead to an increase in PC mass upon cholesterol loading of fibroblasts and macrophages [319-321]. Increase in PC biosynthesis in macrophages is an important adaptive response preventing cholesterol-induced macrophage necrosis [32, 322].

Analysis of the phospholipid molecular species revealed significant changes in acyl chain unsaturation after 24 h of cholesterol loading (***II, Fig. 7***). The concentration of 16:0-SM decreased while that of 24:1-SM increased correspondingly. In case of PC and PE there was a decrease in saturated and monounsaturated acyl chains, whereas the polyunsaturated species increased. The change towards increased unsaturation was observed already after 8 hours of cholesterol loading (data not shown). Taken together, because the acute cholesterol loading also

caused an increase in polyunsaturated fatty acids, this increase was a true regulatory response to cholesterol increase also in NPC cells.

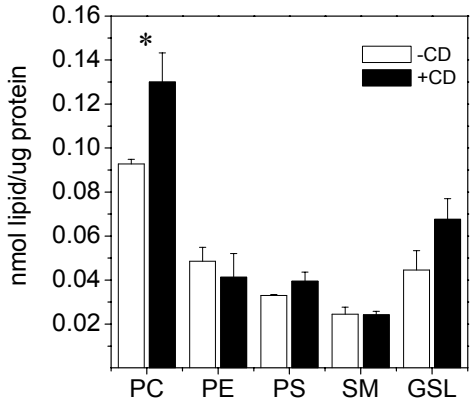

Figure 7. Concentrations of polar lipids in control fibroblasts and fibroblasts loaded with cholesterol using methyl-β-cyclodextrin/cholesterol complexes. Error bars = standard deviation, n = 3, * = $p < 0.05$ between control and NPC.

2.3 Lipid composition of plasma membrane domains incorporated into enveloped viruses

Both control and NPC fibroblasts were infected with the influenza virus or VSV, and the viruses budded to the medium were collected after 24 h of infection. The determined lipid compositions were compared between *i*) viruses and whole cells, *ii*) influenza virus and VSV and *iii*) control cell derived viruses and NPC cell derived viruses.

First, comparison of the lipid class distribution of viruses to that of whole cells revealed that influenza virus was enriched in glycosphingolipids relative to the whole cells (***II, Figs. 1, 4A, B***). In addition, both viruses were enriched in cholesterol relative to the cells, which agrees with the established enrichment of cholesterol on plasma membrane [50-52]. When comparing the phospholipid acyl chain composition of the viruses to that of the whole cell, it was found that polyunsaturated species of PE and PC were depleted from viruses compared to the total cellular lipids (***II, Figs. 2, 5, 6, Table 1***). These data agree with the reported enrichment of saturated fatty acids in the plasma membrane [48, 157].

Second, comparison of the lipid class distribution between influenza virus and VSV showed that the levels of glycerophospholipids and cholesterol were similar in both viruses, but there was a

significant enrichment of glycosphingolipids in the influenza virus (***II, Fig. 4 A, B***). This is consistent with the hypothesis that influenza virus is enriched in raft domains. The phospholipid acyl chain compositions of influenza virus and VSV were similar as shown for PE and PS in **Fig. 8** (***II, Figs. 5, 6***). This result is unexpected because influenza virus would be expected to be enriched in phospholipids with saturated acyl chains as they promote the formation of l_o domains [10, 114, 130, 133]. A possible explanation for this result may be the long infection time used in this study and considered warranted on the basis of previous results [27], but which nevertheless may have undermined the compositional differences between influenza virus and VSV [323].

Third, comparison of the viruses derived from control and NPC cells revealed no significant differences in the concentrations of polar lipids but a significantly higher content of cholesterol in the NPC cell derived viruses (***II, Fig. 4C, D***). This indicates that the plasma membrane of NPC cells contains more cholesterol than that of normal cells. Some previous studies have suggested that plasma membrane of NPC cells may be cholesterol depleted in comparison with normal cells [314, 324, 325]. On the other hand, Lange and coworkers have demonstrated that in NPC cells cholesterol trafficking from late endosomes to plasma membrane is equal to that in normal cells, suggesting that the cholesterol content of NPC cell plasma membrane is normal [326, 327]. One reason for the discrepancy between studies may be that the cholesterol determination methodology used in some of them is based on resistance of NPC cells to sterol-binding antibiotics filipin and amphotericin B [314, 324, 325]. This method, however, may not reflect a reduction in plasma membrane cholesterol content of NPC cells but, for instance, a diversion of the antibiotic to cholesterol-rich endosomes/lysosomes [327].

Significant differences in the phospholipid acyl chain unsaturation between control and NPC cell derived viruses were observed (**Fig. 8**). Both the influenza virus and VSV from NPC cells contained more polyunsaturated PE, PS and PC species than the respective viruses derived from control cells (***II, Figs. 5, 6, Table 1***). The changes towards more polyunsaturated acyl chains were more pronounced in the viral plasma membrane preparations than in whole cells.

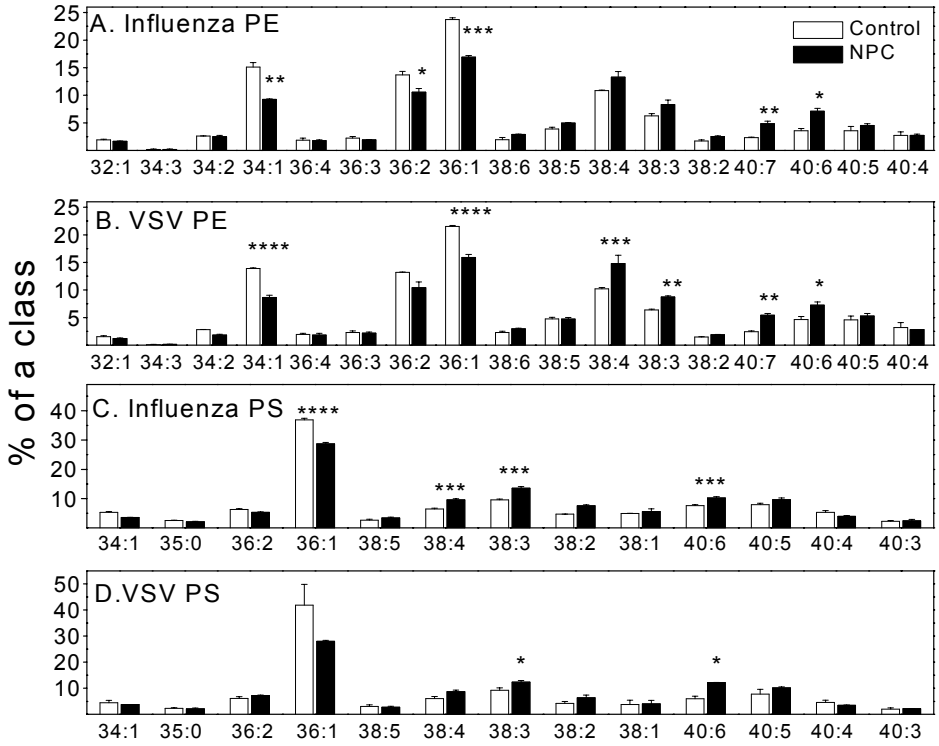

Figure 8. Molecular species composition of PE and PS from influenza virus and VSV grown in control or NPC fibroblasts. PE molecular species in *(A)* influenza and *(B)* VSV, PS species in *(C)* influenza and *(D)* VSV. Error bars = SEM (n = 4-6). **** = $p < 0.001$, *** = $p < 0.005$, ** = $p < 0.01$, * = $p < 0.05$ between values for control and NPC.

2.4 Possible mechanisms for the altered phospholipid acyl chain composition upon increased membrane cholesterol

The increased level of polyunsaturated phospholipid species upon increased membrane cholesterol both in whole cells and in the plasma membrane could reflect a homeostatic regulatory response, because model membranes rich in polyunsaturated PC species have been reported to be more resistant to the stiffening effects of cholesterol [135, 136, 328]. Notably, however, model membrane studies on ordering effects of cholesterol on PC species may not be biologically relevant, because the molecular areas occupied by polyunsaturated PC species are higher than the areas occupied by the corresponding non-PC phospholipids [329]. Therefore, the cholesterol-induced effects on PC order may not reflect the effect of cholesterol in biological membranes which are poor in polyunsaturated PC species.

The mechanism how the increased cholesterol concentration induces increases in the content of unsaturated phospholipids is not known. Decreased membrane fluidity induced by cholesterol may upregulate the synthesis of polyunsaturated fatty acids by enhancing fatty acid desaturases, such as stearoyl-CoA desaturase (SCD) [93]. It has been demonstrated that feeding cholesterol-rich diets to rodents increases the amount of monounsaturated fatty acids [330-332] by inducing SCD gene expression [333-336]. An increase in SCD activity may in fact diminish the amount of rafts in the plasma membrane [337]. Upregulation of SCD alone cannot, however, explain the present increases in polyunsaturates because phospholipid acyl chains derived from essential fatty acids 18:1 (n-6) and 18:2 (n-3), such as PE 40:6 and 40:7 (**Fig. 8**), were also elevated.

3. PARTITIONING OF PYRENE-LABELED PHOSPHO- AND SPHINGOLIPIDS BETWEEN ORDERED AND DISORDERED MEMBRANE DOMAINS (*III*)

Pyrene lipids are useful tools in studying intracellular lipid trafficking and domains. The aim of this study was to elucidate which pyrene labeled lipids partition into ordered domains, and therefore, would be useful probes to study cellular lipid domains. For this purpose, the effect of the length of the labeled acyl chain and the polar headgroup of Pyr$_n$PC, Pyr$_n$SM and Pyr$_n$GalCer (n = 6-14 carbons) on the partitioning of the parent lipid between membrane domains was determined. First, we studied partitioning of these lipids between gel and fluid domains coexisting in bovine brain sphingomyelin (BB-SM) and BB-SM/spin-labeled PC bilayers. Second, partitioning of the probes between l_d and l_o domains in BB-SM/spin-labeled PC/cholesterol bilayers was investigated. The degree of partitioning was determined on the basis of *i*) measurement of excimer to monomer emission intensity ratio (E/M) as a function of temperature or *ii*) quenching of the pyrene monomer fluorescence by spin-labeled PC molecules.

3.1 Gel/liquid domain partitioning of pyrene-labeled lipids as deduced from E/M vs. temperature plots

To measure E/M as a function of temperature we used BB-SM bilayers containing 2 mol% of pyrene lipid to determine partitioning of pyrene-labeled lipids between gel and fluid bilayer

domains [104, 263, 267-269]. BB-SM, which is a mixture of several molecular species, undergoes a gel-to-fluid phase transition between ~15 and 47 °C as shown in **Fig. 9A** (***III, Fig. 1A***) [119, 338]. This transition actually consists of at least three subtransitions due to the acyl chain heterogeneity of BB-SM. The subtransitions at ~36 °C and ~41 °C may be attributed to domains consisting of species with long and saturated acyl chains, while the transition at ~26 °C probably derives from the melting of the species with shorter and/or more unsaturated chains. We interpreted the E/M vs. temperature plot obtained for Pyr_6PC in BB-SM bilayer (**Fig. 9B**) as follows (***III, Fig. 1B, C***) [104, 267-269]. At low temperatures, short-chain (Pyr_6PC) molecules cluster because of their low solubility in gel-state BB-SM domains (**leftmost panel in Fig. 9C**). The exclusion of Pyr_6PC from the BB-SM gel domains can be attributed to a poor fit of the bulky pyrene moiety into the tightly packed lipids in such domains. Upon heating, E/M gradually increases until T_m of BB-SM is reached. At this point, E/M decreases abruptly due to a gradual disappearance of the Pyr_6PC cluster domains upon melting of the matrix lipid (**middle panel in Fig. 9C**). Further increase in temperature results in a monotonous increase in E/M due to increasing lateral diffusion of the pyrene lipid molecules fully dispersed in the fluid BB-SM matrix (**rightmost panel in Fig. 9C**).

The E/M vs. temperature curves obtained for each Pyr_nPC species were markedly dependent on the length of the pyrene-labeled chain (***III, Fig. 2A***). The abrupt decrease of E/M upon heating became less evident with increasing chain length and eventually disappeared. Pyr_nSM (***III, Fig. 2B***) and $Pyr_nGalCer$ (***III, Fig. 2C***) behaved similarly to Pyr_nPC. These results indicated that partitioning of the pyrene lipids to the gel phase domains increases systematically as *n* increases. The gel/liquid partition of the different pyrene-labeled lipids was assessed more quantitatively by using an empirical model (***III, Appendix 1***). The gel vs. liquid partition coefficients ($K_{g/f}$) showed that the probes with a short labeled chain partitioned mainly to the fluid domains at 36°C, while those with a long chain partitioned equally to both types of domains, or even slightly preferred the gel domains (***III, Fig. 4A***), in line with previous studies [267, 269].

A close examination of the E/M vs. temperature curves (***III, Fig. 2, 4A***) indicates that Pyr_nSM may partition somewhat less to the gel domains than Pyr_nPC with equal *n*. A possible explanation for these data is that the pyrene moiety of Pyr_nSM is less deeply inserted in the bilayer than that of corresponding Pyr_nPC. Hoffmann and coworkers have recently proposed that a nitroxide moiety

attached to the acyl chain of SM lies approximately one methylene unit closer to the membrane surface than when the same acyl is attached to the *sn*-2 position of PC, which could be due to different molecular conformations of SM and PC [339]. An alternative explanation would be that the whole SM molecule is less deeply inserted in the bilayer than PC due to its more polar character. Recent computer simulation studies also suggest that SM and PC have different conformations because it was shown that SM species form intramolecular hydrogen bonds and have an increased ordering of hydrocarbon chains compared to PC species [99, 100].

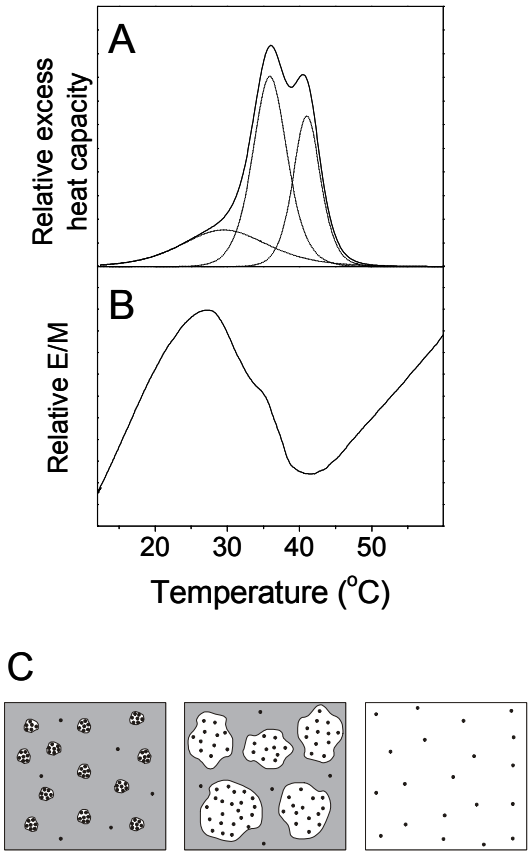

Figure 9. Relationship between E/M vs. temperature curves and phase behaviour of short-chain pyrene lipids. Multilamellar vesicles consisting of 98 mol% BB-SM and 2 mol% of Pyr_6PC lipid were prepared and *(A)* the heat capacity and *(B)* E/M ratio were measured as a function of temperature. *(C)* Relation of the E/M ratio to the distribution of Pyr_6PC below T_m (leftmost panel), at the phase transition region (middle panel) and above T_m of BB-SM (rightmost panel). The grey areas represent gel and white areas fluid domains, while the black dots represent Pyr_6PC molecules.

3.2 Partitioning of pyrene lipids between gel and fluid domains as determined by fluorescence quenching

We also used a fluorescence quenching assay to study partitioning of pyrene labeled lipids between fluid and gel domains [111, 112, 114, 115, 134, 255, 340]. The pyrene-labeled probe was incorporated into a bilayer consisting of 7-SLPC, a spin-labeled quencher lipid with a low T_m, and BB-SM, a lipid with a high T_m. At a temperature below T_m of the unlabeled lipid but above T_m of quencher-labeled lipid, fluid domains consisting mainly of the quencher lipid and gel domains consisting mainly of the unlabeled lipid co-exist. When the ratio of the two lipids is varied, the fractional areas of the fluid-like (quenching) and gel-like (non-quenching) domain will change, which in turn affects probe fluorescence in a manner depending on its partitioning between the two types of domains. A simple equation can then be fitted to the data to obtain $K_{g/f}$ [340].

We first studied quenching of Pyr_6PC and $Pyr_{14}PC$ and also other probes in homogenous, fluid bilayers. It was found that their fluorescence was quenched equally independent of the length of the pyrene-labeled chain in such homogenous bilayers (***III, Fig. 5A***), which is a prerequisite for obtaining reliable partitioning information in segregated bilayers [114]. In contrast, the quenching curves obtained for Pyr_nPC in BB-SM/7-SLPC bilayers, differed markedly from each other; Pyr_6PC and $Pyr_{10}PC$ were quenched more efficiently than $Pyr_{14}PC$ (***III, Fig. 5B***). Analogously, Pyr_6SM and $Pyr_{10}SM$ were quenched more efficiently than $Pyr_{14}SM$, and $Pyr_6GalCer$ more efficiently than $Pyr_{12}GalCer$ (***III, Fig. 5C+D***). These data indicate that the long-chain pyrene-labeled lipids partition preferentially to the gel domains also in BB-SM/7-SLPC bilayers (***III, Appendix 2, Fig. 6***).

This is the first detailed study on how the position of a fluorophore (or other bulky group) along an acyl chain of a phospho- or sphingolipid affects the partitioning of the labeled lipid between coexisting gel/liquid domains. In one study partitioning of two PC species carrying a fluorescent diphenylhexatriene moiety [341] and in another study partitioning of anthroyloxy-labeled fatty acids between gel and fluid domains were studied [112]. Our results indicated that partitioning of Pyr_nPC, Pyr_nSM and $Pyr_nGalCer$ shift in favor of the gel phase when the length of the labeled chain

increases. Plausible explanations for this could be the two things that happen simultaneously: *i*) the effective length of the chain increases and *ii*) the pyrene moiety moves deeper into the bilayer [272].

Regarding the former, i.e. the effective length of the acyl chain, it is likely that the partitioning of the long-chain pyrene lipids to the gel domains is, at least in part, due to the fact that the length of their acyl chains better matches the length of the chains of the lipids forming those domains [112, 133, 134, 254, 255]. Considering the latter, i.e. the effect of pyrene position in the bilayer, it has been shown that the mean bilayer depth of the pyrene moiety attached to a phospholipid acyl chain increases systematically with increasing length of the linked chain [272]. Since in BB-SM bilayers the acyl chains are more ordered close to bilayer surface as compared to the core region [342], pyrene attached to a short acyl chain ($n \leq 8$) will partly reside in the more ordered region of the bilayer. This is expected to significantly perturb the packing of BB-SM acyl chains. When the length of the labeled chain increases, on the other hand, the pyrene moiety moves to the less ordered core region of the bilayer where it is better accommodated than when it is closer to the bilayer surface. Thus, short-chain pyrene-labeled lipids seem to perturb packing of neighboring acyl chains similarly to unsaturated lipids partitioning to the fluid phase [114, 133, 343, 344]. Also computer simulation studies have shown that hydrophobic probes embedded into membranes decrease the order of lipid acyl chains most when they are close to the bilayer surface [273, 274].

The lipid backbone and headgroup structures were also found to affect, although modestly, the distribution of pyrene lipids between gel and liquid domains. Pyr$_n$GalCer seemed to have a somewhat higher affinity for the gel domains than either Pyr$_n$PC or Pyr$_n$SM when $n > 8$ (***III, Fig. 4, 6***). This is consistent with the recent data obtained for lipids containing an indolylstearic acid residue [134]. The higher gel domain affinity of the Pyr$_n$GalCer derivatives could derive from that these species are better accommodated in such closely packed systems due to their smaller headgroup [345]. However, the data of Wang and Silvius indicate that there is no simple relationship between the headgroup size and partitioning between fluid and gel domains [134].

3.3 Partitioning of pyrene lipids between liquid-ordered (l_o) and liquid-disordered (l_d) domains

Partitioning of the pyrene lipid between l_o and l_d domains was assessed by using the fluorescence quenching method. The quenching curves obtained for Pyr$_n$PC in BB-SM/7-SLPC/cholesterol bilayers showed that Pyr$_6$PC is quenched significantly more efficiently than either Pyr$_{10}$PC and Pyr$_{14}$PC, thus indicating that this short-chain species partitions significantly less to l_o domain than those with a longer chain (***III, Fig. 7A***). The Pyr$_n$SM and Pyr$_n$GalCer derivatives behaved similarly to Pyr$_n$PC, i.e., the short-chain species appeared to partition less into l_o domains than the long-chain ones (***III, Fig. 7B+C***). However, the Pyr$_{14}$SM and Pyr$_{12}$GalCer species were less quenched at low 7-SLPC mole fractions than Pyr$_{14}$PC, suggesting that they favor the BB-SM-rich domains more than the PC derivative.

The partition coefficients [114] obtained showed that, in general, the affinity for the l_o domains increases with the length of the pyrene-labeled chain (***III, Fig. 8***). In agreement with present data, also phospho- and sphingolipids with another type of fluorophore attached to a short acyl chain were found to partition largely into l_d domains whereas an increase in acyl chain length increased the affinity for l_o domains [114, 115, 130, 186]. Pyr$_n$PC species, however, are an exception from this rule, since Pyr$_{14}$PC partitioned less to the l_o domains than Pyr$_{10}$PC (at higher 7-SLPC mole fractions). A possible explanation could be that in the l_o domains the pyrene moiety of Pyr$_{14}$PC interacts unfavorably with the molecules in the opposite leaflet. Therefore, Pyr$_{14}$PC could have a lower affinity for such domains than the species with a somewhat shorter chain, e.g., Pyr$_{10}$PC. The absence of such anomalous chain-length dependency in BB-SM/7-SLPC bilayers (***III, Fig. 4, 6***) could relate to the fact that the gel domains are thicker than the l_o domains [201, 202] and thus the Pyr$_{14}$PC chain does not reach the opposite monolayer in BB-SM gel domains. Consistent with this reasoning, studies carried out with other labeled lipids showed that an optimal chain-length exists for partitioning into l_o domains [114, 134]. The fact that partitioning of Pyr$_n$SM to l_o domains increased steadily with *n* with could be due to a shallower position of the pyrene as compared to Pyr$_n$PC with equal *n* (see above). However, it cannot be excluded that also other factors, such as the proposed higher affinity of SM for cholesterol [70] play a role here.

The headgroup also influenced the partitioning of pyrene lipids into l_o domains, since the partitioning of long-chain pyrene lipids into l_o domains increased in the order PC < SM < GalCer (***III, Fig. 8***). This order agrees with that obtained previously for other fluorescent lipids [115, 134]. A possible explanation for the preferential partitioning of glycosphingolipid analogues into l_o domains could be that the glycosyl residues of these species contribute to their affinity for these domains [115].

4. DISTRIBUTION OF PYRENE-LABELED SPHINGOMYELINS IN LIVING CELLS

The domain partitioning data obtained with model membranes (***III***) indicated that pyrene-labeled lipids should be useful tools to study the role of membrane domains in lipid sorting and trafficking. To study the behaviour of pyrene-labeled lipids in cells, we introduced pyrene-labeled sphingomyelins (Pyr_nSM) of different chain lengths into human fibroblasts. We followed their metabolism at different time points, studied the cellular distribution by fluorescence microscopy and the domain partitioning by detergent insolubility.

4.1 Introduction of Pyr_nSMs into human fibroblasts and their metabolism therein

Pyrene-labeled sphingomyelins of varying chain lengths were introduced into the plasma membrane of human fibroblasts during a 5-minute pulse by using γ-CD as a carrier and the cells were then chased for 1, 5, 17 or 41 hours in the absence of serum. The uptake of Pyr_nSM into cells determined by HPLC analysis was ~5 pmols of pyrene lipid/μg protein. Minor differences in the amount of lipid incorporated into cells reflected differences in the labeling efficiency between experiments and the different analogues. In comparison, the concentration of endogenous SM in fibroblasts was 26 pmol/μg protein (***II***). Up to 5 hours of chase, the majority of Pyr_4SM and $Pyr_{12}SM$ remained unmetabolised, as ~75% of Pyr_4SM and ~80% $Pyr_{12}SM$ was still intact (**Fig. 10 A+B**). The major metabolic product of Pyr_nSMs was ceramide [280-282]. Interestingly, at long chase times differences in the rate of ceramide formation were observed

between the different pyrene lipids, in line with previous data [281], because at 41 hours, ~20% of Pyr$_4$SM, and ~50% of Pyr$_{12}$SM had been hydrolysed to corresponding ceramides (**Fig. 10 A+B**). Pyr$_{16}$SM hydrolysis into the corresponding ceramide (data not shown) was similar to Pyr$_{12}$SM hydrolysis. This indicated that the short-chain derivative was catabolised less efficiently than the long-chain derivatives.

To determine whether Pyr$_n$SMs were hydrolysed into ceramide by an acidic lysosomal or a neutral sphingomyelinase (SMase), the pulse-chase experiments were also performed with Niemann-Pick A (NPA) fibroblasts which are defective in the lysosomal SMase. HPLC analysis indicated that the hydrolysis of Pyr$_4$SM and Pyr$_{12}$SM into ceramide was virtually absent in NPA cells, signifying that the acidic lysosomal SMase was mostly responsible for the degradation of these pyrene labeled SMs in fibroblasts (**Fig. 10 C+D**). The present results are in agreement with previous data obtained for Pyr$_{12}$SM and short-chain pyrene labeled sphingomyelins whose hydrolysis was shown to be significantly reduced in NPA fibroblasts compared to control fibroblasts [281]. It has also been shown, however, that a minor part of short-chain pyrene labeled SMs can be degraded into ceramides also by a neutral non-lysosomal SMase [281, 282]. Also, NBD-SM has been shown to be partially hydrolysed by a neutral SMase [257].

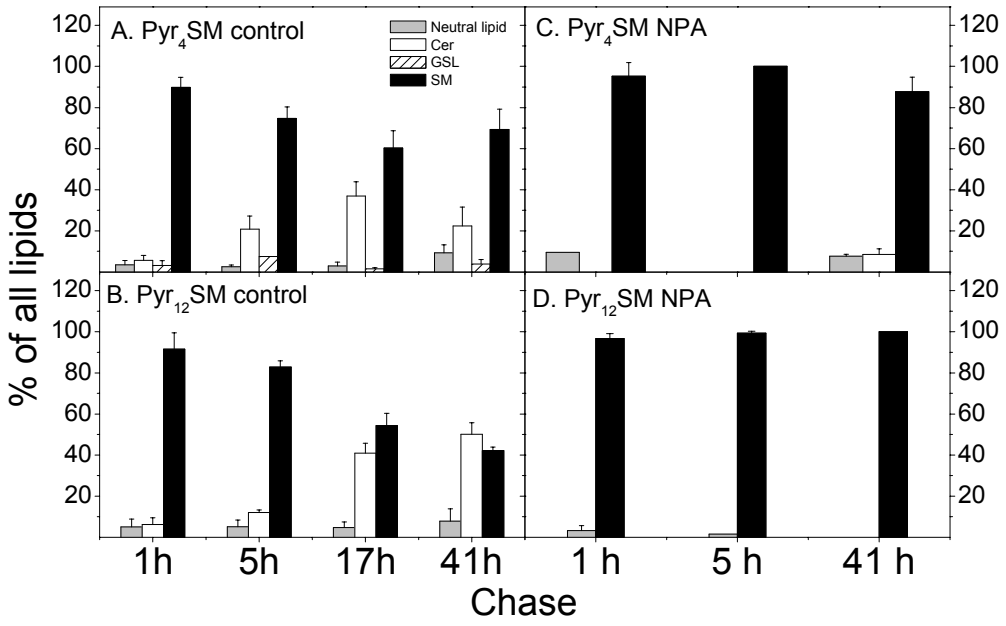

Figure 10. Metabolism of Pyr$_4$SM and Pyr$_{12}$SM in human fibroblasts. Degradation was determined for Pyr$_4$SM and P$_{12}$SM in *(A+B)* control and *(C+D)* NPA fibroblasts by HPLC with fluorescence detection (see experimental procedures). The values are expressed as % of the quantified lipids and they represent an average of 2-4 experiments with duplicate samples. Error bars = standard deviation.

4.2 DRM association of Pyr$_n$SMs

Triton X-100 insolubility of pyrene labeled SMs was used as a measure of their partitioning to ordered domains in cellular membranes. Fibroblasts were first labeled with ^{3}H-choline chloride for 24 hours to metabolically label PC and SM species followed by labeling with Pyr$_n$SM and 1h of chase. DRMs were subsequently isolated by flotation in an Optiprep-Triton X-100 gradient. ^{3}H-choline distribution between collected Optiprep fractions (**Fig. 11B, inset**) was determined. The distribution of total ^{3}H-choline labeled lipids in the gradient corresponded to that of PC, indicating that the Optiprep-Triton X-100 gradients correctly reflected the solubility of cellular lipids. DRM association of Pyr$_n$SMs was determined by fluorescence spectroscopy. Pyr$_{16}$SM was by far the derivative most enriched in DRMs, as ~60-70% was recovered in the top fractions of the gradient (**Fig. 11A**). Pyr$_{12}$SM was ~30% insoluble, whereas Pyr$_4$SM was

almost completely detergent-soluble. Thus, the chain-length dependent domain partitioning of pyrene labeled lipids demonstrated in model membranes (***III***) was also supported at the cellular level. Interestingly, BODIPY-labeled SM and LacCer were both detergent-soluble (data not shown). ESI-MS analysis of the major fibroblast phospholipids indicated that SM was 60-70 % enriched in DRMs as expected, whereas PC and PE were mostly TX-100 soluble (**Fig. 11B**). Thus, the insolubility of $Pyr_{16}SM$ resembled the insolubility of endogenous SM.

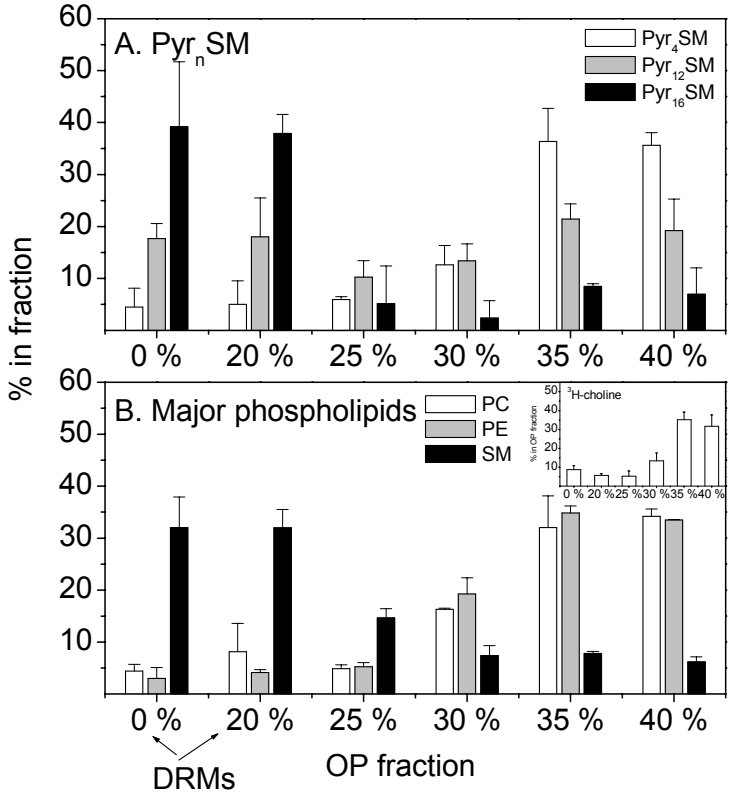

Figure 11. Association of* Pyr$_n$SMs *and fibroblast lipids with DRMs as determined by flotation in Optiprep-1% TX-100 gradient. *(A)* Distribution of Pyr$_n$SMs after 1h of chase. *(B)* ESI-MS analysis of the distribution of major fibroblast phospholipid classes, n = average of 2-3 experiments. *(B, inset)* Distribution of total ^{3}H-choline containing lipids after metabolic labeling for 24 hours, n = 8. Error bars = standard deviation.

4.3 Intracellular distribution of Pyr$_n$SMs

The distribution of Pyr$_n$SM monomer fluorescence in fibroblasts was visualised by fluorescence imaging. Fibroblasts were labeled and chased with Pyr$_n$SM as outlined above. Pyr$_n$SMs initially incorporated onto the plasma membrane from where they were endocytosed rapidly as shown for Pyr_4SM and $Pyr_{12}SM$ in **Fig. 12A+C**. To better visualise the intracellular structures, the plasma membrane fluorescence was selectively quenched by TNP-LPE (**Fig. 12 B+D)** [277]. By

imaging the cells before and after TNP-LPE addition the fraction of fluorescence inside the cell and in the plasma membrane could be determined. At 15 minutes of chase the fraction of monomer fluorescence inside the cells was 0.23 (±0.03, n = 12 cells) for Pyr$_4$SM and 0.27 (±0.03, n = 12 cells) for Pyr$_{12}$SM, i.e. 70-80% of the lipid analogues was on the plasma membrane. As the chase time was increased the fraction of Pyr$_n$SM fluorescence inside the cell increased. At 2 hours of chase, the fraction of monomer fluorescence inside the cells was 0.37 (±0.07, n = 18 cells) for Pyr$_4$SM and 0.42 (±0.07, n = 18 cells) Pyr$_{12}$SM, i.e. ~60% of the fluorescence was in the plasma membrane. Part of the intracellular fluorescence of Pyr$_4$SM at 2 hours co-localised with early endosomal antigen-1 (EEA1) in punctate structures (**Fig. 12 E+F**) suggesting that Pyr$_4$SM was transported to early endosomes. Fibroblasts labeled with Pyr$_{16}$SM were also imaged (data not shown), however, due to its low level of incorporation into the cells the interpretation of these images was problematic.

Detergent insolubility of Pyr$_n$SMs was also studied by fluorescence imaging [158]. Fibroblasts were labeled and chased with Pyr$_4$SM or Pyr$_{12}$SM as described above (**Fig. 13 A+C**), extracted with 1% Triton X-100 for 30 minutes on ice, fixed and imaged. We found that the cell surface area remained largely labeled by Pyr$_{12}$SM (**Fig. 13B**), apart from the extracted holes present in each cell. In contrast, Pyr$_4$SM was completely extracted by the detergent (**Fig. 13D**). These data support the detergent extractability of Pyr$_n$SMs as analysed by the Optiprep-gradient.

In conclusion, γ-CD provides a rapid and simple method to introduce even the more hydrophobic Pyr$_n$SM species into living cells and to image their distribution therein by fluorescence microscopy. The cellular metabolism of Pyr$_n$SMs is relatively slow allowing one to study the probe distribution by fluorescence imaging at time points where no significant degradation has yet occurred. The domain partitioning or Pyr$_n$SMs as determined by their DRM association supports the data obtained in model membranes (***III***), i.e. the analogues with long labeled acyl chains partition preferentially into ordered domains also in living cells. By using lipid analogues with different domain-partitioning preferences information on the role of lipid domains in cellular processes will be obtained. The possibility to visualise both monomer and excimer fluorescence simultaneously enables one to obtain E/M ratio images, which should provide valuable information on the *local* pyrene lipid concentration. In particular, E/M images

before and after treatments that perturb raft lipid balance on the plasma membrane (e.g. depletion of SM by exogenous neutral SMase or depletion of cholesterol by cholesterol-sequestering agents) is expected to provide information on changes in membrane fluidity and organisation.

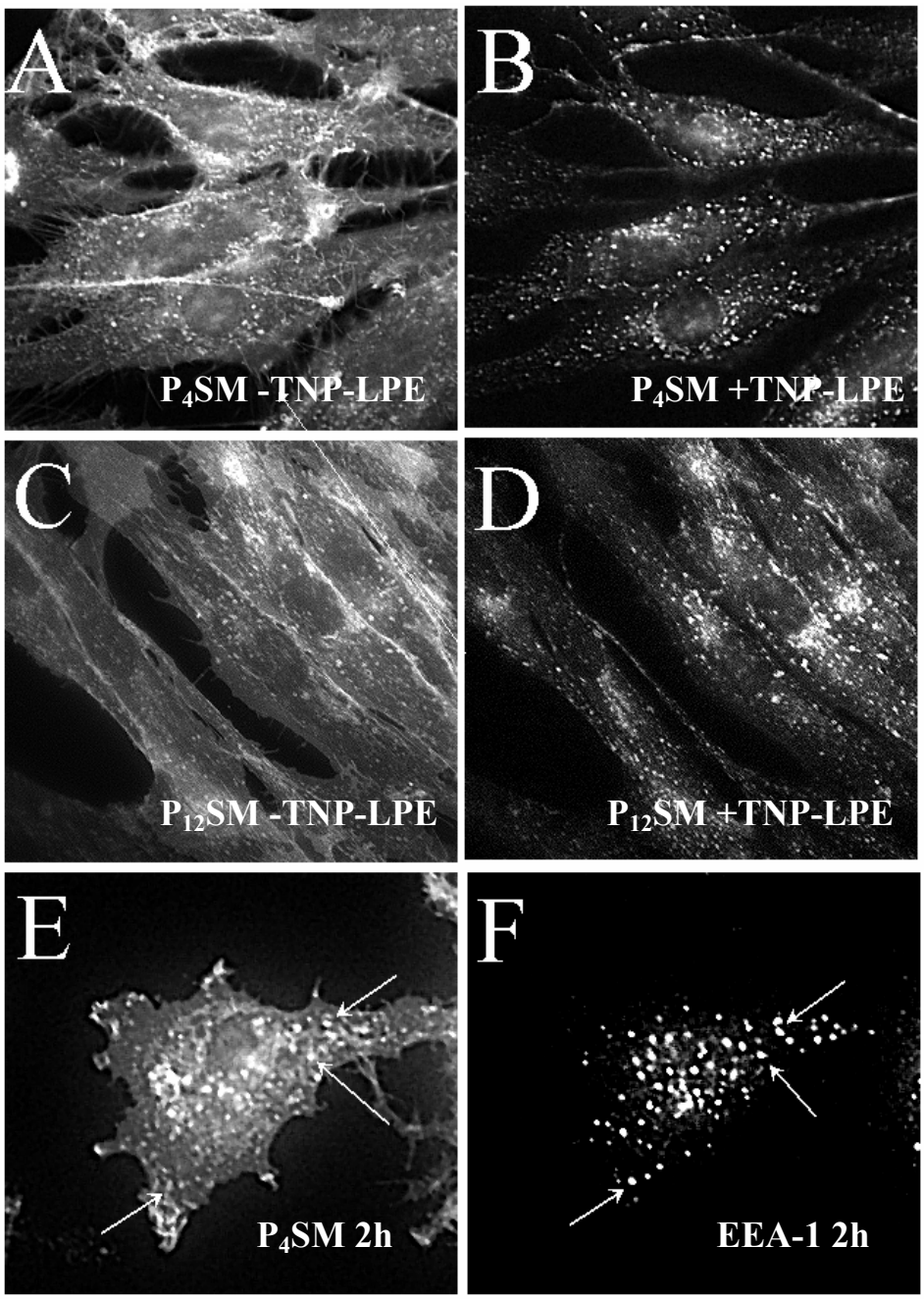

Figure 12. Fluorescence imaging of Pyr$_n$SMs in human fibroblasts. *(A)* Pyr$_4$SM monomer fluorescence after 15 min chase, *(B)* the same image as in A after selective quenching of plasma membrane fluorescence by TNP-LPE. *(C)* Pyr$_{12}$SM monomer fluorescence after 15 min chase, *(D)* the same image as in C after quenching of plasma membrane fluorescence by TNP-LPE. *(E)* Pyr$_4$SM after 2h chase in fixed cells, *(F)* EEA-1 localisation in the same cell as in E, punctata showing co-localisation of Pyr$_4$SM and EEA-1 are indicated with arrows.

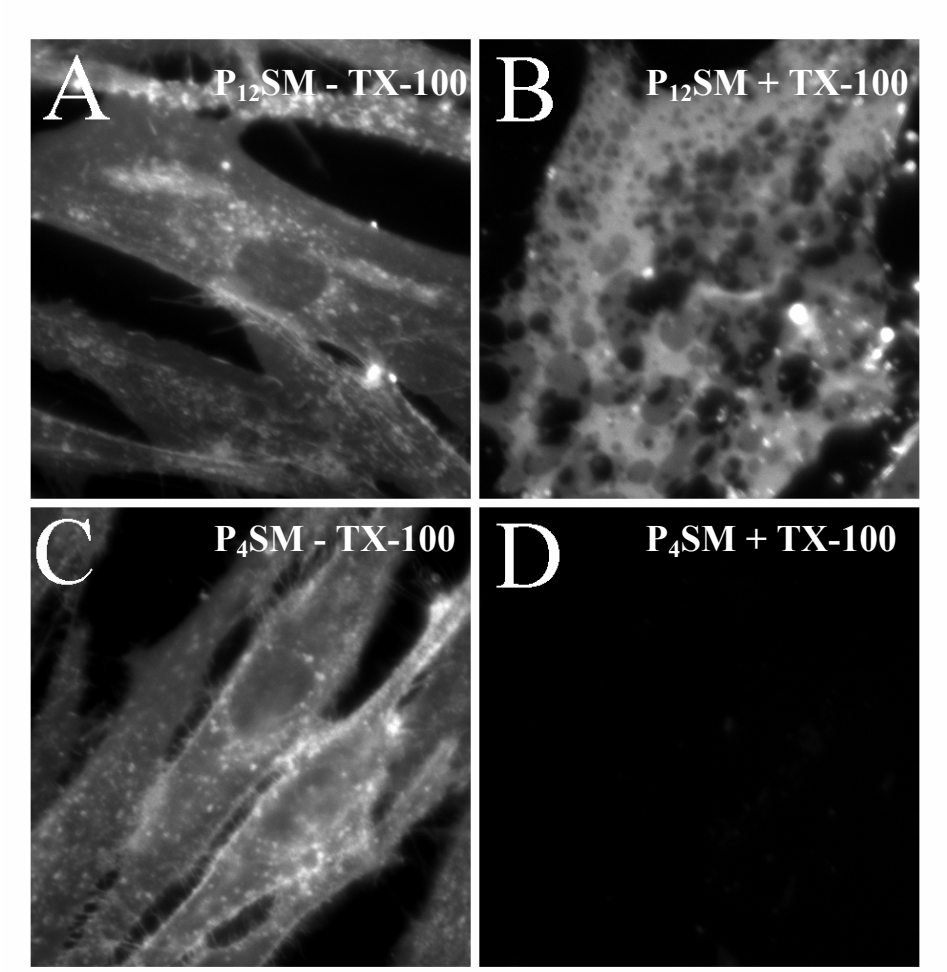

Figure 13. Cold Triton X-100 extractability of $Pyr_{12}SM$ and Pyr_4SM in fibroblasts. After labeling cells were chased for 15 min, imaged, extracted with 1% Triton X-100 on ice for 30 minutes, washed, fixed with 4% paraformaldehyde and the same dish was imaged again. A dish labeled with $Pyr_{12}SM$ and imaged *(A)* before and *(B)* after TX-100 extraction, a dish labeled with Pyr_4SM and imaged *(C)* before and *(D)* after TX-100 extraction. Detergent extraction after fixing of the cells gave essentially the same result. The intensity scale in images before and after the detergent treatment is the same.

CONCLUSIONS

The key results of the present study are:

1) Several factors influence the instrument response in electrospray ionisation mass spectrometric (ESI-MS) analysis of phospholipids. Among other things, phospholipid acyl chain length and unsaturation, polar headgroup as well as total lipid concentration have a significant effect on instrument response. Reliable quantitative data on phospholipid compositions can be achieved by ESI-MS by including several internal standards for each lipid class in the lipid extract.

2) ESI-MS analysis of fibroblast plasma membrane domains enveloped into budded viruses show that glycosphingolipids are enriched in influenza virus thought to bud from ordered domains, compared to VSV budding from disordered domains. Both chronic and acute cholesterol loading of fibroblasts increase the content of polyunsaturated fatty acids in major phospholipid classes of plasma membrane domains as well as of whole cells. This could reflect a regulatory mechanism in cells to maintain an adequate level of membrane fluidity when the cholesterol concentration of the membranes is increased.

3) Partitioning of pyrene-labeled PC, SM and GalCer (Pyr_nPC, Pyr_nSM and Pyr_nGalCer) between ordered and disordered domains in model membranes is strongly influenced by the length of the labeled acyl chain. The long-chain pyrene-labeled analogues partition preferentially into ordered domains and the short-chain analogues favor fluid domains.

4) Pyrene-labeled lipids partitioning into ordered and disordered domains can be introduced into cells with γ-cyclodextrin. Their domain partitioning therein appears to depend on the length of the labeled acyl chain similarly as in model membranes. Therefore, pyrene-labeled lipids are useful tools to study properties of membrane domains as well as their role in lipid sorting and trafficking in cells.

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