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Review

Lipids in exosomes: Current knowledge and the way forward

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ABSTRACT

Lipids are essential components of exosomal membranes, and it is well-known that specific lipids are enriched in exosomes compared to their parent cells. In this review we discuss current knowledge about the lipid composition of exosomes. We compare published data for different lipid classes in exosomes, and what is known about their lipid species, i.e. lipid molecules with different fatty acyl groups. Moreover, we elaborate on the hypothesis about hand-shaking between the very-long-chain sphingolipids in the outer leaflet and PS 18:0/18:1 in the inner leaflet, and we propose this to be an important mechanism in membrane biology, not only for exosomes. The similarity between the lipid composition of exosomes, HIV particles, and detergent resistant membranes, used as lipid rafts models, is also discussed. Furthermore, we summarize knowledge about the role of specific lipids and lipid metabolizing enzymes on the formation and release of exosomes. Finally, the use of exosomal lipids as biomarkers and how the lipid composition of exosomes may be of importance for researchers aiming to use exosomes as drug delivery vehicles is discussed. In conclusion, we have summarized what is presently known about lipids in exosomes and identified issues that should be taken into consideration in future studies.

Contents

Abbreviations: BMP, bismonoacyl glycerophosphate; CE, cholesteryl ester; CHOL, cholesterol; Cer, ceramide; DAG, diacylglycerol; DRM, detergent resistant membrane; FCS, fetal calf serum; Gb3, globotriaosylceramide; GLC, gas liquid chromatography; HexCer, hexosylceramide; HG, hexadecylglycerol; LacCer, lactosylceramide; LBPA, lysobisphosphatic acid; MVB, multivesicular body; MS, mass spectrometry; PA, phosphatidic acid; PC, phosphatidylcholine; PC O/P, PC ethers (alkyl or alkenyl); PE, phosphatidylethanolamine; PE O/P, PE ethers (alkyl or alkenyl); PG, phosphatidylglycerol; PLD2, phospholipase D2; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; SMase, sphingomyelinase; TAG, triacylglycerol; TLC, thin layer chromatography.

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1. Introduction

Exosomes are small vesicles (40–150 nm in diameter) released from cells after fusion of the multivesicular bodies (MVBs) with the plasma membrane [1,2]. The biogenesis of exosomes can be regarded as a three-step process: (a) biogenesis of MVBs, (b) transport of MVBs to the plasma membrane, and (c) release of the intraluminal vesicles of the MVBs as a consequence of fusion of MVBs with the plasma membrane. Exosomes are considered as one of three main types of extracellular vesicles (Fig. 1). The other types of extracellular vesicles are considered to be larger than exosomes, i.e. vesicles budding off from the plasma membrane (typically in the size range 100–1000 nm) and apoptotic bodies (500–2000 nm) formed by blebbing of membranes of apoptotic cells [3,4]. Exosomes have a complex composition including proteins, nucleic acids, lipids and other metabolites [5,6]. During recent years there has been much focus on exosomes. How are they made and secreted to the extracellular environment? What is their specific composition? Which physiological and pathological roles do they have? Can exosomes be used as biomarkers or as vehicles for drug delivery? These aspects have been reviewed in several articles during the last years [1,2,7–12]. However, the lipid-related aspects of exosomes have not obtained sufficient attention. A 10-year old review article summarized what was then known about the lipid composition of exosomes [13], but at that time there were very few mass spectrometry (MS) data published for exosomes. More recent reviews have focused on the role of exosomes in lipid metabolic diseases and cell-to-cell communication [14,15].

In the present review we will critically discuss the current knowledge about the lipid compositions of exosomes and about the lipid classes and species (lipids with different fatty acyl groups) that are enriched from cells to exosomes. In addition, the effect of lipids and lipid metabolizing enzymes on the formation and release of exosomes will be summarized. Furthermore, based on the current focus on the potential clinical applications of exosomes, we will discuss the use of exosomal lipids as biomarkers and how lipids can affect the possible use of exosomes as vehicles for drug delivery.

Finally, we would like to highlight that there are several similarities between the lipid composition of exosomes, HIV particles and detergent resistant membranes (DRMs), isolated from cells as models for lipid rafts. Thus, we have added a discussion about similarities and differences of the lipid composition of exosomes, HIV particles and DRMs in the present review.

Before discussing the lipid data reported for exosomes, we will give some general comments on the methods used for exosome isolation and the uncertainty of the lipid analyses used in these studies. We have decided to use the same nomenclature for describing the different types of vesicle preparations as those used in the original articles, as it in most cases is difficult to evaluate if another nomenclature would be better to describe these preparations.

2. Methodological aspects related to the study of the lipid composition of exosomes

2.1. Methods used to isolate exosomes

Exosomes are isolated from different cell types grown in culture as well as from biological fluids such as plasma and urine, and the reported lipid composition can vary as discussed below. There are several possible explanations to the variations of the lipid composition of exosomes discussed in this review. Different cell types and growth conditions, as well as the methods used for isolation and lipid analyses may all contribute to the results discussed.

Starting with cell growth conditions, we have shown that different cell densities may change the lipid composition and intracellular trafficking [16]. Also the lipid composition of the cell medium, mainly fatty acids, may be important for the lipid composition of the cells [17], and thus most probably for exosomes. Also other substances present in serum can play a role. In particular, the lipid composition of exosomes is likely to change after removal of serum from the medium or its replacement with ultracentrifuged serum, something that is done in several exosome studies due to the presence of exosomes in fetal calf serum (FCS).

The most commonly used method to isolate exosomes so far is ultracentrifugation, and this method has been used in the studies presented in Table 1. Ultracentrifugation may, however, result in co-isolation of exosomes, lipoparticles and lipid droplets, which then will result in a lipid composition different from that obtained if only exosomes with a membrane just consisting of the normal bilayer structure were present. The removal of FCS during the collection of exosomes from cell lines would avoid this problem, but as described above, growing cells for several hours (days) without serum may have consequences. Moreover, our experience is that the so-called exosome-free serum contains particles with a similar size as exosomes, and these particles may be of lipid nature and could co-purify with exosomes. Co-isolation of lipoproteins with extracellular vesicles is particularly a problem in blood plasma samples [18]. Other methods used to isolate exosomes are filtration, size-exclusion chromatography, immunoaffinity capture, polymer-based precipitation and microfluidics [19–22]. All these methods have pros and cons due to the heterogeneity of extracellular vesicles and their coexistence in cell culture media or biological fluids with potential lipid structures such as lipoproteins. Especially, one should be careful about conclusions drawn following analyses of exosomes purified by using columns or affinity methods that may result in purification of only a subpopulation of exosomes originally present.

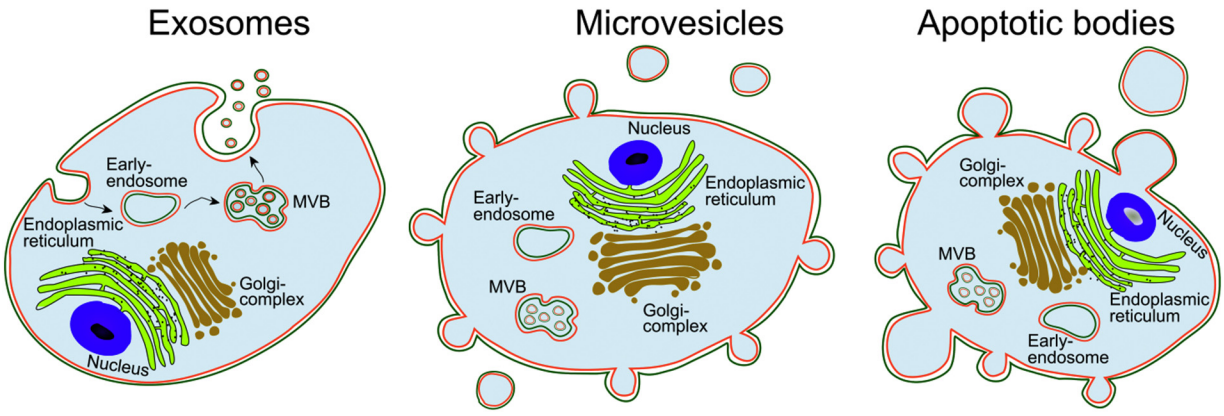

Fig. 1. Extracellular vesicles are released by different mechanisms. Exosomes are released after fusion of MVB with the plasma membrane. Microvesicles are released by directed budding of the plasma membrane. Apoptotic bodies released by apoptotic dying cells are also considered a type of extracellular vesicles. The figure is reprinted from the PhD degree of Santosh Phuyal, University of Oslo, with permission from the author.

Table 1
Lipid content in exosomes and enrichment from the originating cells.

	PC-3 cells [34]		PC-3 cells + HG [43]		Oli-neu cells [35]		B-lymphocytes [36]		Mast cells [37]		Dendritic cells [37]		Reticulocytes [38]		Prosta.[a] 100 nm [39]	Prosta.[a] 50 nm [39]	Urine [40]	Nematodes [41]
Lipids	%	Factor	%	Factor	%[b]	Factor	%	Factor	%[c]	Factor	%[c,d]	Factor	%	Factor	%	%	%	%[e]
CHOL	43.5	2.3	59	1.7	43	2.3	42.1	3.0	15	1.0	?	?	47	1.03	54.8	54.1	63	7
SM	16.3	2.4	9.1	2.0	8.2	1.5	23.0	2.3[f]	12	2.8	20	2.2	8.4[f]	1.31	28.6	14.3	11.7	3
PC	15.3	0.31	10.8	0.33	26.7	0.67	(20.3)[g]	(0.76)[g]	28	0.66	26	0.6	23.5	1.03	2.3	1.3	2.7	4
PS	11.7	2.1	6.9	1.2	14.9	3.0	(20.3)[g]	(0.76)[g]	(16)[g]	(1.2)[g]	(19)[g]	(1.6)[g]	5.9	0.92	7.1	5.4	13.2	15
PE	5.8	0.55	1.1	0.21	10.9	1.0	(14.6)[g]	(0.7)[g]	24	1.08	26	1.13	12.7	0.84	0.6	0.3	<LOQ	13
PE O/P	3.3	1.2	4.7	0.81			(14.6)[g]	(0.7)[g]									4.6	47
DAG	1.5	1.5	1.1	0.92													0.1	
PC O/P	0.81	0.40	0.7	0.28														8
HexCer	0.76	3.8	2.3	2.1	?	2.0									5.6	23.3	1.9	
Cer	0.32	1.3	0.7	1.2	?	3.3											0.1	
PG	0.17	0.17	0.1	0.07														
PA	0.16	1.8	0.1	0.33			(20.3)[g]	(0.76)[g]										
PI	0.13	0.13	0.3	0.16			(20.3)[g]	(0.76)[g]	(16)[g]	(1.2)[g]	(19)[g]	(1.6)[g]	2.4	1.1	1.1	1.3		
LacCer	0.12	3.0	0.7	1.8													0.8	
LysoPI	0.09	2.3	–	–														
LysoPE	0.09	1.3	–	–														
CE	0.08	0.38	–	–													0.3	
Gb3	0.02	2.0[h]	–	–													1.1	
Out/In	1.47		1.49		1.35		?		1.15		1.22		1.52		4.1	5.6	1.03	0.20
Lipid analysis	MS		MS		MS		TLC		TLC/GLC		TLC/GLC		TLC		LC-MS	LC-MS	MS	MS
Exosome prep.[i]	SFM + SUC		SFM + SUC		SFM + SUC + SG		uFCS + SUC + SG + immunocapture		uFCS + SUC		uFCS + SUC		uFCS + SUC		SUC + SEC + SG	SUC + SEC + SG	SUC	SFM + SUC

%: Percent of total lipid quantified.
Factor: Factor of enrichment from cells to exosomes.
Out/In: Sum of sphingolipids and lipids with the phosphocholine head group divided by all other lipids reported (except CHOL).
LOQ: Limit of quantification.
[a] Prosta = Prostasomes.
[b] These data are recalculated by assuming same level of CHOL as in [34]; the absolute amounts of CHOL, Cer and HexCer were not reported.
[c] Recalculated from their data.
[d] CHOL not reported; the sum for the other lipid classes is 100% (including LysoPC not included in this table).
[e] Recalculated from their data; lyso ethers and lyso acyl lipids are not shown.
[f] Sum of SM and the ganglioside GM3.
[g] Sum for all classes shown in parentheses and having the same numbers.
[h] Enrichments of other GSLs (GM1, GM2, GM3 and GD1) are shown in Fig. 1; due to lack of standards these GSLs could not be quantified as the other lipid classes.
[i] Exosome prep.: Methods used to isolate the exosome preparations. SFM: serum free medium; SUC: sequential centrifugation; SG: sucrose gradient; uFCS: ultracentrifuged fetal calf serum; SEC: size exclusion chromatography.

When evaluating the lipid composition of exosome preparations one should keep in mind that CE and TAG, the lipids that form the hydrophobic core of lipid droplets and lipoproteins, are not present in cellular membranes [23]. Therefore, if relatively large amounts of these lipids are obtained in exosome preparations, it is likely that lipids droplets or lipoprotein particles have been co-isolated with exosomes. Lipid droplets could be co-isolated with exosomes if cells have been ruptured. Also, lipid droplets can be included in autophagic vesicles [24], and it has been shown that exosomes can be co-isolated with autophagic material in cells where secretory autophagy is induced [25].

In conclusion, development of better methods to purify and characterize the different types of extracellular vesicles is certainly needed. For the present discussion it is important to stress that it is not easy to evaluate the purity of the exosome samples used in several of the studies discussed in the present review. We have, however, commented upon such issues when the lipid analyses indicate that the preparations contain "impurities", i.e. that the lipids do not fit well with a bilayer membrane structure formed by selection of lipids from the plasma membrane or endosomal membranes.

2.2. Lipid analyses

A key issue regarding comparison of the thin layer chromatography (TLC), gas liquid chromatography (GLC) and mass spectrometry (MS) results discussed in the present review is the uncertainty of the data reported. It is not easy to give general statements about this as it is not only dependent on the method used, but also on the competence of the people using these sometimes very specialized methods, and the purity of the samples analyzed. It should however be mentioned that it has for many years been well-known that different lipid species may contribute to different MS signal intensities [26,27]. We refer to review articles where several aspects of quantification and reproducibility of different MS analyses have been discussed [28–31]. We believe that in the analyses performed in our own studies the uncertainty is 5–15% for most species analyzed, but the uncertainty may be larger for some minor species. We think readers inexperienced with interpreting lipid data such as those shown in Table 1 should keep in mind that the uncertainty could be larger than 15%.

2.3. Asymmetric distribution of lipids in the two leaflets of exosomal membranes

It is well established that there is an asymmetric distribution of the lipid classes in the plasma membrane, such that SM, other sphingolipids and PC (at least most of it) can be expected to be in the outer leaflet, whereas all other classes are expected to be mainly in the inner leaflet [23]. The asymmetry of membrane bilayers may be changed by enzymes such as flippases, floppasses and scramblases [32,33], and there have been discussions about if and to which extent breakdown of the asymmetry of the exosome bilayer may occur, e.g. by PS flipping to the outer leaflet. We refer to articles demonstrating asymmetry in the lipid distribution in the exosome membrane in Section 4.

As the ratio of the area of the outer leaflet to the inner leaflet will depend upon the size of small vesicles such as exosomes, we have

calculated the theoretical ratios between the areas of the outer and inner leaflets for vesicles of different sizes (assuming the thickness of the bilayer to be 5 nm). The surface area of a sphere is $4\pi r^2$, which means that the ratio for the outer to the inner leaflet of exosomes with a diameter of 70 nm will be $35^2/30^2 = 1.36$. This ratio increases for smaller vesicles. Of relevance for the discussion below, vesicles with a diameter of 50–100 nm will have an area ratio of the outer to the inner leaflet of 1.56–1.24.

3. Lipids in exosomes

3.1. Enrichment of lipid classes from cells to exosomes

The lipid content of exosomes and their enrichment factors from cells to exosomes have been reported in several studies (Table 1). The most detailed study so far, including quantification of approximately 280 lipid species from 18 lipid classes, is our own study with the prostate cancer cell line PC-3 [34]. However, as shown in Table 1, several studies have described the percent of different lipid classes in cells and exosomes in several cell types such as Oli-neu [35], human B-cells [36], mast cells RBL-2H3 [37], dendritic cells [37], and vesicles released during in vitro maturation of guinea pig reticulocytes [38]. In addition, Table 1 contains information about exosome preparations from biological fluids where the lipid content of the donor cells is not known. In particular, data of two different sizes (100 nm and 50 nm) of prostasomes (i.e. exosomes expected to be released from prostate cells) isolated from human seminal fluid [39], exosomes isolated from human urine [40], and exosomes secreted by a nematode parasite [41] are included.

Several of the studies listed in Table 1 show 2–3 times enrichment from cells to exosomes for cholesterol, SM, glycosphingolipids and PS. In contrast, exosomes generally contained less PC (mol% of total lipids) than their parent cells, and only small changes were reported for PE in most studies. The changes in the relative amounts of lipid classes from cells to exosomes for all 22 lipid classes (the 18 listed in Table 1 and four classes of gangliosides) quantified in PC-3 cells [34] are shown in Fig. 2; these data show several lipid classes so far reported for exosomes only in this study. The mol% of all lipid classes in these exosomes are given in Table 1, and the mol% of these classes in the parent cells can be calculated using the enrichment factors. It should be mentioned that there exists an additional study of the lipid composition of PC-3 cells and five other prostate cell lines (including one nonmalignant cell line) as well as exosomes released from these cell lines [42]. Those data show an enrichment from cells to exosomes of SM (2.5–3.7 times) and glycosphingolipids (1.8–3.3 times), whereas all phospholipid classes were grouped and do not add much to the present discussion. It is interesting to see that PC-3 cells treated with hexadecylglycerol (HG), a precursor for ether phospholipids, gave similar enrichment factors from cells to exosomes as untreated cells [43], despite of the changes addition of this precursor causes in the lipidome of both exosomes and PC-3 cells (Table 1).

The description of the lipid composition of exosomes released from Oli-neu cells [35] is the second most detailed study comparing the lipid content of exosomes and their parent cells. The changes from cells to exosomes for Oli-neu cells show several similarities with those of PC-3 cells, but the exosomes from the Oli-neu cells show less enrichment of SM and a much higher enrichment of Cer (this is further discussed in Section 6).

Whereas the studies mentioned so far have been based upon lipid analyses using modern MS technology, the following studies discussed in this chapter were performed using older methods like TLC and GLC. The group of Stoorvogel was the first to include MS analyses (in addition to TLC) to study the lipid content of exosomes [36]. Using human B-cells they were also the first to describe a large enrichment of cholesterol and sphingolipids from cells to exosomes, and the similarities between the lipid classes enriched in exosomes and in detergent-resistant membranes (DRMs). It is, however, difficult to compare their data with the data obtained using MS analyses, as the lipid classes in the B-cell study were quantified using a TLC method where PC, PS, PI and PA co-migrated in one band. Similarly, SM and the ganglioside GM3 co-migrated and thus had to be quantified together (Table 1).

We face somewhat similar challenges when looking into the lipid composition of mast cells and dendritic cells [37], where PS and PI were quantified together, i.e. one class found to be highly enriched in exosomes from PC-3 cells and one class found to be higher in the PC-3 cells than in exosomes (Fig. 2). Another issue that makes it difficult to interpret these data is the lack of cholesterol measurements in the dendritic cell study, and the surprising information that the cholesterol content (mol% of total lipids) was almost the same in mast cells and their exosomes (Table 1).

Table 1 also shows the lipid composition of reticulocytes and their exosomes. This study from 1989 was in fact the very first to describe the lipid content of exosomes and their parent cells [38]. As shown in Table 1, these data show only a slight enrichment of SM and a small decrease in the mol% of PE, whereas for all other lipid classes (including cholesterol) the data for cells and exosomes are within the analytical uncertainty of the method used. It should be noted that these cells

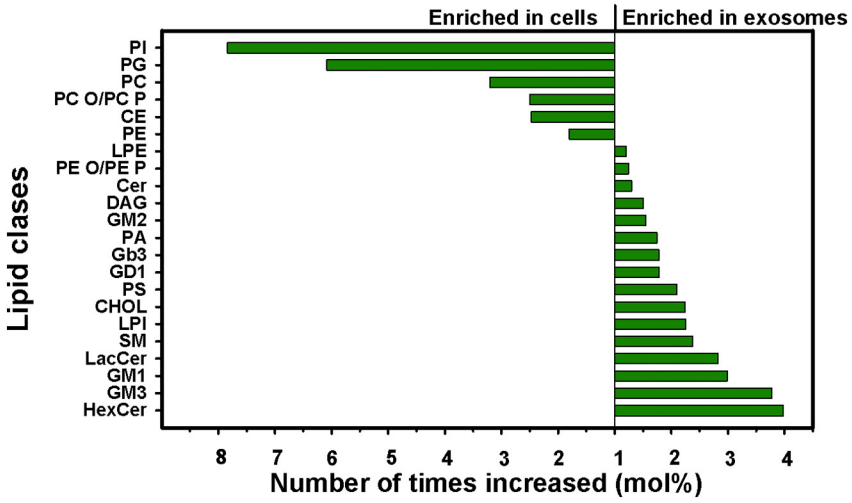

Fig. 2. Enrichment of lipid classes in PC-3 cells or exosomes released from these cells calculated as mol% of lipids. This figure is reprinted from Llorente et al. [34] with permission from Elsevier.

have very high cholesterol content, and a further enrichment in the released exosomes cannot be expected. One should also be aware of that MVBs fusing with the plasma membrane of reticulocytes have been reported to bear markers of early, not late, endosomes [44]. These cells also lose their transferrin receptors as they are transformed into erythrocytes, and one may therefore speculate that there is a different sorting of lipids into exosomes in reticulocytes than in other cells. In another study with reticulocytes (not listed in Table 1), exosomes were purified after 2, 4 and 7 days of differentiation and showed major changes in the main lipid classes measured, i.e. PE, PI + PS, PC and SM [45].

In a recent study more than 500 lipid species were measured in the colorectal cancer cell line LIM1215 and its exosomes [46]. This study is not included in Table 1 because quantification of the relative abundance of different lipid species was performed by comparing their MS signals with the internal standard PC14:0/14:0. Thus, only relative abundance of species in the cells and exosomes were reported, and it is therefore not easy to compare these data with those shown in Table 1. By comparing the lipid ion abundance per μg protein for the different lipid classes and calculating the enrichment from cells to exosomes (note that this is not enrichment of lipid classes from cells to exosomes, but enrichment of lipid classes per μg protein in the two samples) some surprising patterns were observed. We estimated the enrichment factors for the lipid classes reported by the authors to be: TAG (x24), SM (x12), DAG (x11), CE (x5.7), cholesterol (x4.9), and PC, PE and PS (all x1.7). Furthermore, for PS and PE, the lysolipids were much more enriched from cells to exosomes than the diacyl species. These results are thus very different from those reported by others, and the very high enrichments of TAG and CE might indicate the presence of lipoparticles and/or lipid droplets in the exosome preparations.

Haraszti et al. [47] recently published major differences in the lipid composition for exosomes, microvesicles and parental cells for U87 glioblastoma cells, Huh7 hepatocellular carcinoma cells, and human bone marrow-derived mesenchymal stem cells (MSCs). They state that 1961 lipid species were identified in these samples. It is, however, difficult to evaluate their results as they are presented as summary numbers only. They reported a similar content of SM and PS in exosomes and parental cells (percent of lipids), which is contrary to most other reports discussed above, whereas the levels of PC and PI are more similar to those discussed above for these classes.

As mentioned in Section 2.3, we have used the lipid data shown in Table 1 to calculate the ratio of lipid classes expected to be in the outer and inner leaflets by assuming the same asymmetry as in the plasma membrane. These ratios are shown in the third row from the bottom of Table 1, and for the studies discussed so far they fit reasonably well with the theoretical values calculated for vesicles with a diameter of 50–150 nm (i.e. ratios of 1.56–1.24).

In addition to the lipid classes discussed above, it is of interest to investigate if BMP (bismonoacyl glycerophosphate), also called LBPA (lysobisphosphatic acid), a lipid present in membranes of the intraluminal vesicles of MVBs, is present in exosomes [23]. To our knowledge, there are only two studies where BMP analyses have been performed on exosomes. In the first study, BMP was reported to be below the detection limit of the analysis [36]. In the other study, BMP was reported to account for 0.8 and 1.2 mol% of total phospholipids for exosomes and cells, respectively [37]. It was concluded in both studies that BMP is not transferred on to exosomes, but most likely present in the intraluminal vesicles of MVBs that fuse with lysosomes. This view is in accordance with what most scientists believe is the main destination of BMP, as it has been described to contribute to lysosomal stability and integrity and to be an essential cofactor for sphingolipid catabolism in lysosomes [48,49]. We agree that available data support the idea that BMP is not present in exosomes, at least not in large amounts. We think, however, that it would be interesting to analyze BMP in other exosome preparations and their parent cells using modern MS technology before concluding if BMP is present in exosomes, perhaps in a cell type-dependent manner.

3.2. Composition of exosomes isolated from biological fluids or secreted from a nematode

The lipid composition of extracellular vesicles in biological fluids is less well characterized and has so far only been described for extracellular vesicles from seminal fluid (prostasomes) and from urine (Table 1). In terms of prostasomes, two prostasome preparations (100 and 50 nm in diameter, respectively) purified from human seminal fluid have been analyzed by MS [39]. These results deviate considerably from other exosome preparations (Table 1). The content of SM and HexCer is so high that it is difficult to understand how these lipid compositions can fit with a bilayer structure. In order to form vesicles of these sizes with bilayer structures, it is necessary that e.g. all PC and approximately ¼ of the sphingolipids (i.e. the SM and HexCer content shown in Table 1) are in the inner leaflet in contrast to the rest of the sphingolipids. Thus, these prostasomes are either very different from all other exosomes published, or these vesicle preparations prepared from human seminal fluid contain more than just vesicles with a normal membrane bilayer.

The lipid composition of prostasomes purified from human [50], horse [51], and boar [52] semen were obtained by using TLC methods to quantify the relative amounts of SM, PC, and PE and the sum of PS and PI (co-migrating). These three studies all show very high cholesterol contents and a lipid composition more similar to the exosome data discussed above than to the two prostasome preparations [39] shown in Table 1. Also the calculated ratios of lipid classes expected to be on the outer and inner leaflets are more similar to the values discussed above for exosomes, i.e. 1.86, 1.04 and 1.22 for human, horse and boar, respectively. It should be noted that HexCer, an important constituent in the two prostasome preparations listed in Table 1, was not measured in these earlier studies.

The lipid composition of exosomes purified from human urine has recently been described [40] (Table 1). These results were obtained following quantification of 107 lipid species. The most surprising observations are: (a) the very high content of cholesterol, on the borderline of what has been reported in model membranes [53]; (b) the finding that PS 18:0/18:1 is the main lipid species after cholesterol (see discussion below about lipid species); and (c) that all PE species detected were identified as PE ethers. It is not known why PE ethers are selected into the exosomes isolated from urine, but we think this is interesting also in light of the extremely high amounts of PE ethers in exosomes released from nematodes [41], and also the high content of PE ethers in HIV particles [54] discussed below.

Remarkably, extracellular vesicles are also released by parasites where they appear to contribute to the infection process [55]. Very recently, exosomes secreted from a nematode parasite in mice were described to have a very surprising lipid composition [41]. These exosomes had a remarkable high content of PE plasmalogens, i.e. alkenyl ethers (47 mol%), and very low contents of cholesterol (7 mol%) and SM (3 mol%). The authors propose that nematodes maintain exosome structure and stability by the high concentration of plasmalogens compensating for the very low levels of cholesterol and SM. Nematodes are not believed to synthesize sterols, but to obtain sterols through the diet; and the sphingolipid synthesis is not very well described in this organism [41].

3.3. Selection of lipid species into exosomes

Cells contain thousands of different molecular lipid species, and there has been an increasing number of examples describing specific cellular functions for one single lipid species [56]. Although the exosomal amounts of different lipid classes has been determined in several studies, there are only a few studies where molecular lipid species have been analyzed and even fewer where they have been quantified. In our study of PC-3 cells [34], approximately 280 species from a total of 18 lipid classes were quantified. This is to our knowledge the only

study showing quantitative data for more than a few molecular lipid species found in both exosomes and their parent cells.

Most groups that have compared the saturation level of fatty acyl groups in lipids in exosomes with that in parent cells agree that there is an enrichment of phospholipid species with two saturated fatty acyl groups. For PC, this is mainly due to an increase in PC 14:0/16:0 and 16:0/16:0 [34,35]. However, we think it is important to stress that exosomes contain abundant monounsaturated fatty acyl groups. PC 16:0/18:1 and PC 16:0/16:1 were the two dominating PC species in exosomes from PC-3 cells [34], PC 34:1 and PC 32:1 were among the most common species in exosomes from Oli-neu cells [35], and PC 34:1 accounted for approximately 60% of the PC species in both prostasome preparations listed in Table 1 [39].

The lipid data for exosomes from PC-3 cells demonstrate a remarkable enrichment of several species with C18:1 in the *sn-2* position in different lipid classes. Most remarkably was the enrichment and total amount of PS 18:0/18:1 in PC-3 cells [34], and in urinary exosomes PS 18:0/18:1 was the main species after cholesterol [40]. PS 36:1 contributed approximately 80% of the total PS species in the two prostasome preparations [39], and it was the only PS species listed in the B-cell study [36]. Also for other lipid classes there was a remarkable enrichment of species containing C18:1 in exosomes released from PC-3 cells, as such enrichment was observed for PE 18:0/18:1, PE 16:0/18:1, PE 18:1/18:1, PI 18:0/18:1, PI 16:0/18:1, PI 18:1/18:1 and DAG 18:0/18:1. Note that species with the combination 18:0/18:1 was enriched in all classes of PS, PE, PI and DAG. Also in the two prostasome preparations PE 34:1 and PE 36:1 were the main PE species, and they together constituted approximately half of the PE species [39]. Thus, monounsaturated fatty acyl groups gave a major contribution to the lipid species of PS, PC and PE species both in exosomes from PC-3 cells and in the two prostasome preparations.

Sphingolipids, and especially the main sphingolipid SM, are certainly important for the structure of exosomes. Although there was a 2.4-fold enrichment of SM species from PC-3 cells to their exosomes, there were only minor changes in the relative contribution of the main SM species, i.e. SM d18:1/16:0, SMd18:1/24:0 and SM d18:1/24:1, which constituted approximately 35%, 20% and 20%, respectively, of the total SM species in exosomes [34]. Somewhat similar data were reported for the SM content of the two prostasome preparations shown in Table 1, where the main SM species was the N-amidated 16:0 (35–40%) followed by almost equal amounts (15%) of 20:0, 22:0 and 24:0, and with 24:1 as the most abundant (5–10%) unsaturated fatty acyl group [39]. Thus, the main difference between these data sets was that prostasomes have less very-long-chain and unsaturated SM species (C24:1).

As mentioned above, exosomes released from nematodes have a very different composition of lipid classes compared to other exosome preparations [41]. Also the species composition within the different lipid classes was very different in the nematode exosomes. The most surprising observation was that a PE ether with 36 carbon atoms and two double bonds (36:2) constituted 25% of the total lipids analyzed. Moreover, the content of phospholipids with two unsaturated fatty acyl groups was high as PC 36:2, PS 36:2 and PE 36:2 were all the dominating species of these lipid classes, whereas species with 36 carbon atoms and 1 or 3 double bonds were also among the most common species for PC, PS and PE. Thus, unsaturated phospholipid species and especially the PE ether (36:2) are extremely important building blocks of the nematode exosomes.

4. Hand-shaking of lipids between the two membrane leaflets

In our study of PC-3 cells and their released exosomes [34], we noticed that cholesterol, the very-long-chain SM (mainly SM d18:1/24:0 and SM d18:1/24:1) and PS 18:0/18:1 (which is the main PS species constituting approximately 40% of the total of PS species in these exosomes) were enriched to a similar extent from cells to exosomes. The enrichment factors observed, 2.2, 2.4 and 2.7, respectively, are probably within the analytical uncertainty. This made us speculate that these lipids are sorted together, and that there may be a "hand-shaking" between the very-long-chain SM in the outer leaflet and this PS species in the inner leaflet (note that species with 24 carbon atoms should be able to penetrate deep into the inner leaflet [57]). Moreover, if one compensates for the different surface area of exosomes with a diameter of 70 nm, the PS 18:0/18:1 in the inner leaflet could theoretically cover approximately 80% of the area covered by the very-long-chain SM in the outer leaflet. By including PE 18:0/18:1 in these calculation, PS 18:0/18:1 plus PE 18:0/18:1 could theoretically cover 95% of the area covered by the very-long-chain SM in the outer leaflet. We have also investigated the lipid composition of PC-3 cells treated with the ether lipid precursor HG [43]. The addition of this ether lipid precursor caused, as discussed above, several changes to the lipidome of both cells and exosomes, but still the ratio of PS 18:0/18:1 to the very long-chain SM was in the same range in these exosomes as those isolated from untreated cells. Finally, recent analyses of urinary exosomes from 15 prostate cancer patients and 13 healthy volunteers showed that PS 18:0/18:1 in the inner leaflet could occupy 78 ± 18% (mean ± SD) and 66 ± 22% of the area occupied by the very-long-chain sphingolipids in the outer leaflet in patients and controls, respectively [40].

Based on these data we performed molecular dynamic simulation studies to estimate the transmembrane coupling (interdigitation) between the long-chain (C16:0) or very-long-chain (C24:0) SM in the outer leaflet and different phospholipids in the inner leaflet in the presence or absence of cholesterol [57]. These simulation studies revealed that the very-long-chain SM gave a much stronger interdigitation than the long-chain SM. The largest interdigitation was obtained between the very-long-chain SM d18:1/24:0 in the outer leaflet and PS 18:0/18:1 in the inner leaflet, and the interaction between these two species was the only one that was found to increase in the presence of cholesterol. There has during recent years been much discussion about how cholesterol is distributed in the membrane leaflets [58]. In our simulation study, the highest interaction between the two leaflets was found when the cholesterol content was slightly higher in the outer than the inner leaflet. Taken together these results and the lipidomic data, support the idea of a selective handshaking between the very-long-chain sphingolipids and PS 18:0/18:1 (Fig. 3), and we believe this interaction is an important mechanism in membrane biology, not only for exosomes (see discussion in [57]). Fig. 3 shows an illustration of the lipid bilayer of exosomes based upon these quantitative analyses [34] and the hypothesis about hand-shaking between the very long-chain sphingolipids in the outer leaflet and the PS 18:0/18:1 in the inner leaflet.

The present hypothesis about handshaking between the very-long-chain sphingolipids and PS 18:0/18:1 is based upon the assumption that PS (or most of it) is found in the inner leaflet of exosomes, in the same way as in the plasma membrane. In some studies of extracellular vesicles PS is described to be present also in the outer leaflet, as it is detected with Annexin 5. It is well known that PS is found in the outer leaflet of activated blood cells, apoptotic bodies and microparticles or microvesicles released from plasma membranes, and that such exposure of PS functions as an "eat me" signal for macrophages, such that vesicles or cells with PS on their surface are removed from circulation [59]. We refer to a recent review covering the possibilities of PS being important for uptake of certain exosomes into target cells [15].

When thinking about the physiological role of exosomes, it is unlikely in our opinion that they expose PS in general since they would then be removed from circulation by macrophages, and they would be unable to bring signaling molecules to different cells and tissues. A study showing different integrins to be responsible for extracellular vesicles to home into lung or liver [60] is in agreement with this view. Although several studies report binding of e.g. Annexin 5 to exosomes, thus indicating that at least some of the exosomes have PS in the outer leaflet, we have not seen any study convincingly demonstrating the presence of PS on the outer leaflet of exosomes just after secretion from cells. It would

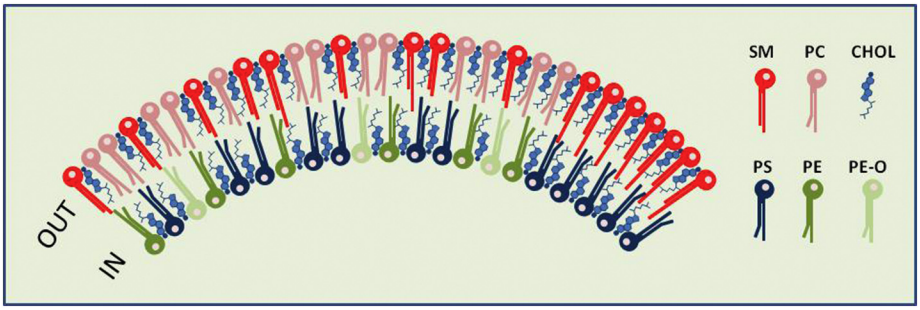

Fig. 3. Illustration of the lipid bilayer of exosomes based upon quantitative lipidomic data for exosomes released by PC-3 cells [34]. The number of lipids (excluding cholesterol) shown in the outer (29) and inner (22) leaflet is close to the ratio of 1.36 for the outer and inner surface of exosomes with an outer diameter of 70 nm. The lipid composition of the membrane in the illustration is based on the data shown in Table 1, i.e.16 SM (one of these should have been HexCer to completely match the published data), 13 PC, 12 PS, 6 PE, 3 PE O and 39 molecules of cholesterol (assuming a close to symmetric distribution of cholesterol between the two leaflets). In the right part of the membrane, a possible handshaking between the very-long-chain sphingolipids in the outer leaflet and PS 18:0/18:1 in the inner leaflet in the presence of cholesterol is illustrated. In the rest of the membrane, the lipids are distributed more or less evenly. Nine out the 16 SM molecules shown contain a very-long-chain N-amidated fatty acyl group in accordance with the data published [34].

be very interesting to know the percentage of exosomes that presumably expose PS, and to investigate whether the exposure of PS is a result of storage, or if it can be observed just after secretion of exosomes. Furthermore, it is of great interest to determine the fraction of total exosomal PS that becomes exposed. Such information can probably be obtained by benefiting from the binding of PS to Annexin 5 [61] or Tim4 [62]. We think this is an essential issue to consider for future studies of exosomes. Importantly, the question of PS exposure in the outer leaflet has been addressed in two recent studies. In a study with microvesicles (expected to be a mixture of exosomes and vesicles shedding off from the plasma membrane) isolated from human bone marrow mesenchymal stem cells, it was reported that PS was exposed on the surface of vesicles isolated from hypoxic cells, but not from non hypoxic cells [61]. In another study with mesenchymal stem cells it was reported that these cells secreted three different types of extracellular vesicles with a size of 50–100 nm. Interestingly, only one of the three types was able to bind Annexin 5, and the Annexin 5 binding vesicles did not contain typical exosomal markers such as CD9, CD81, ALIX and TSG101 [63]. Thus, the results so far indicate that exosomes in general do not expose PS.

5. Comparison of lipids in exosomes, HIV-particles and DRMs

Several authors have commented upon similarities in enrichment of lipids from cells to exosomes, virus particles and DRMs, as all these particles are enriched in cholesterol and SM. We will here discuss such similarities and differences by comparing the lipid data discussed above for exosomes with those reported in some key publications describing the lipid content of virus particles and DRMs. Although all these particles have less PC (mol% of total lipids) than their parent cells, the PC class has relatively more saturated species. Some data for comparison of the lipid composition of virus particles are shown in Table 2, where also data for exosomes from PC-3 cells have also been included for comparison.

To our knowledge, the lipid analyses of HIV particles released from HeLa cells and MT-4 cells provide the most detailed lipid data published for virus particles [54]. The lipid content of these HIV particles and the enrichment of the lipid classes from cells to particles show remarkable similarities with data from exosomes released from PC-3 cells (Table 2). However, HIV particles contain 15 mol% more PE ethers (plasmalogens) and 10 mol% less of cholesterol than PC-3 exosomes. Also when looking into the enrichment of species composition from cells to HIV particles there are large similarities with PC-3 cells and exosomes. PC 34:1 is the dominating PC species and it is enriched in HIV particles as PC 16:0/18:1 is in PC-3 exosomes. The second most abundant PC species in the HIV particles (PC 36:2) contributes much less to the PC species in the HIV particles than in the parent cells as PC 18:1/18:1 does in PC-3 exosomes. The largest enrichment in HIV particles compared to their parent cells was observed for PC 32:0 (enrichment factors of 4.3 and 2.7 in HeLa and MT-4 cells, respectively), whereas a 4.1-fold enrichment was observed for PC 16:0/16:0 (the largest enrichment of all PC species) in PC-3 exosomes. There was no remarkable enrichment of lipid species in the other lipid classes although some selection of species was observed for HIV particles as described above for exosomes.

Due to the hypothesis about hand-shaking discussed above, we looked more closely into the values for PS 36:1 (PS 18:0/18:1 in exosomes) and the very-long-chain SM species (containing 40 or 42 carbon atoms) in the HIV particles. PS 36:1 was the dominant PS species in the two HIV particles and their parent cells. HeLa cells had a distribution of PS species somewhat similar to HEp-2 cells. By assuming these HIV particles to have a diameter of 120 nm [64], PS 36:1 in the inner leaflet could theoretically cover 60% of the area covered by the very-long-chain SM species in the outer leaflet. MT-4 cells have a very different composition of PS species (although PS 36:1 is the dominant PS

Table 2
Lipid data for PC-3 exosomes, HIV particles and detergent resistant membranes (DRMs).

Lipids	PC-3 exosomes [34]		HIV particles; HeLa cells [54]		HIV particles; MT4 cells [54]		DRMs; KB cells [67]		DRMs; KBC cells [67]	
	%[a]	Factor	%	Factor	%	Factor	%	Factor	%	Factor
CHOL	43.5	2.3	33.1	1.9	32.7	1.7	24.0	1.9	33.5	2.3
SM	16.3	2.4	10.3	2.8	15.5	1.7	15.9	1.4	15.2	1.5
PC	15.3	0.31	11.4	0.40	6.2	0.28	14.6	0.65	13.0	0.66
PS	11.7	2.1	9.8	1.7	14.6	2.3	3.0	3.5	2.4	3.0
PE	5.8	0.55	8.9	0.58	5.6	0.39	12.0	0.85	10.3	0.84
PE O/P	3.3	1.2	17.9	1.7	20.9	2.2	21.9	1.3	17.9	1.3
DAG	1.5	1.5								
PC O/P	0.81	0.40								
HexCer	0.76	3.8	2.2	1.5	0.4	2.0				
Cer	0.32	1.3	0.1	0.33	0.1	0.50				
PG	0.17	0.17	0.2	0.17	0.7	1.4				
PA	0.16	1.8					0.7	1.3	0.9	2.3
PI	0.13	0.13	1.0	0.18	1.0	0.12	7.9	0.74	6.9	0.76
Out/In	1.47		0.71		0.55		0.67		0.73	
Methods	MS		MS		MS		MS		MS	

%: Percent of total lipid quantified.
Factor: Factor of enrichment from cells to exosomes, from cells to HIV particles or from cells to DRMs.
Out/In: Sum of sphingolipids and lipids with the phosphocholine head group divided by all other lipids reported (except CHOL).
[a] Same data as reported in Table 1, but the following lipid classes were not included in this table as they were not measured neither in the HIV particles nor in the DRMs: DAG, PC O/P, LacCer, LysoPI, LysoPE, Cer and Gb3.

species also in these cells and their HIV particles), both preparations contain much less PS 34:1 and much more of the polyunsaturated PS 40:5 and PS 40:6 than HeLa and PC-3 cells. For MT-4 cells the ratio of PS 36:1 to the very long chain SM species was so high that PS 36:1 could cover an area of the inner leaflet being 50% larger than that covered by the very-long-chain SM species in the outer leaflet. We wonder if the high amount of PS 36:1 somehow might compensate for the very high amount of the polyunsaturated PS species in MT-4 cells and their HIV particles. The high level of PS 36:1 suggests that there is a handshaking mechanism also in these particles.

The high content of PE ethers and relatively low amount of cholesterol in the HIV particles [54] is interesting in light of the recent data for exosomes from nematodes discussed above, as Simbari et al. speculated that the high content of PE ethers in nematode exosomes could be necessary to compensate for the very low amounts of cholesterol and sphingolipids [41]. It should be noted that the values calculated for the expected ratios of the outer to inner leaflet lipids for the HIV particles and the exosomes from nematodes (Tables 1 and 2) are all very low, and these values decrease with an increasing mol% of the PE ethers. In order to make these lipid compositions fit into a membrane bilayer it is necessary that a rather large percent of these ether lipids are present in the outer leaflet.

DRMs are preparations isolated from cells treated with different detergents (often 1% Triton X-100 at 4 °C). Such preparations have been commonly used to study lipid rafts [65,66]. We have selected to compare the lipid data discussed above for exosomes and HIV particles with those reported for DRM preparations from KB and KBC cells [67], as these are the most detailed lipid analyses we are aware of for such preparations. As shown in Table 2, there are several similarities between these studies, although both DRM preparations and their parent cells contain higher amounts of PI, PE, PE O/P (especially alkenyl ethers, often called plasmalogens), and considerably lower amounts of PS. A closer look at the composition of lipid species in the different preparations shows an upconcentration of the very-long-chain SM species and no enrichment of PC 16:0/16:0 in both DRM preparations, in contrast to exosomes and HIV particles. Intriguingly, the ratio of lipids expected to be in the outer and inner leaflet is much lower for DRMs than could be expected for a bilayer structure, although these ratios are in the same range for the DRMs and HIV particles listed in Table 2.

Lipid studies of other DRM preparations clearly show that DRMs prepared from different cells or using different procedures may have a very different lipid composition. Thus, analyses of DRMs from MDCK cells show a large enrichment of PC 16:0/16:0 [68], and analyses of DRMs from bovine photoreceptor rod outer segment membranes [69] show large enrichments of C16:0 in most lipid classes in contrast to the DRM preparations described above (those listed in Table 2). Moreover, van Gestel and coworkers [70] recently published interesting lipid data for DRMs isolated from human sperm and two epithelial cells lines. They concluded that unsaturation and chain length of fatty acyl groups of the phospholipids are almost similar in the DRMs and cells from which they were isolated. Moreover, these DRMs contained substantial amounts of polyunsaturated phospholipids. They found cholesterol and SM to be enriched in all DRMs, but to a cell-specific molar ratio. Remarkably, extraction of cholesterol from cells with 5 mM methyl-β-cyclodextrin did reduce the cholesterol level in DRMs, but did not affect their composition and amount of phospholipids. When higher concentrations (over 10 mM) methyl-β-cyclodextrin were used, an overall lipid depletion from DRMs was observed, rather than a specific extraction of cholesterol. Also interestingly, all three DRM preparations appeared to have multilamellar vesicular structures as monitored by negative staining electron microscopy analyses.

Several studies show that the lipid and protein content of DRMs depend to a large extent on the method used for their preparation. Thus, different results are obtained when using various detergents, and both the detergent concentration, the temperature used during the isolation, the time used for isolation, as well as the buffer solution and the ions present give DRM preparations with different protein and lipid composition [65,66,71–73]. This has made several investigators to conclude that DRMs are not purified membrane rafts, but lipid structures formed in an equilibrium between the detergents and the total cell lipids (the solubility of different lipids is probably important) at the conditions used, or even aggregates formed by "co-precipitation" of lipids into artificial structures [65,66,74]. Some DRM preparations have also been reported to contain considerable amounts of CE or TAG [68], which may indicate that lipid droplets are isolated together or "co-precipitate" with DRMs.

6. Lipids and lipid metabolizing enzymes involved in formation and release of exosomes

Several lipids and lipid metabolizing enzymes have been shown to play a role in the formation and release of exosomes. Trajokovic et al. reported that inhibition or siRNA-mediated depletion of neutral sphingomyelinase (nSMase) resulted in reduced secretion of exosomes from Oli-neu cells [35]. They proposed that this effect may be due to the formation of ceramide microdomains in areas with high concentrations of sphingolipids, followed by their coalescence into larger ceramide-rich domains promoting membrane budding. Thus, the authors expect the cone-shaped ceramide formed by removal of the large head group (phosphocholine) from SM to be the driver of the nSMase effect. Later, a similar effect of nSMase was reported in the human embryonic kidney cell line HEK293 [75] and in T-cells [76]. These results seem to have created the general opinion that formation of ceramide is essential for exosome secretion. However, removal or inhibition of nSMase do not affect exosome release in all cell lines tested [77–79]. A possible explanation may be that nSMase has different subcellular localizations, resulting in ceramide formation at different locations [80,81]. Furthermore, in PC-3 cells there was no effect on exosome secretion by inhibiting ceramide formation by fumonisin B_1, an inhibitor of de novo synthesis of ceramide [79]. Also inhibition of glucosylceramide synthase, i.e. the first enzyme in the synthesis of glycosphingolipids, did not significantly change the amount of secreted exosomes in PC-3 cells, but modified the protein composition of the secreted exosomes by a so far unknown mechanism [79]. Thus, the effect of ceramide on exosome secretion is not universal and more information is needed to understand why inhibition of nSMase gives different effects in various cell lines. Lipidomic analyses of Oli-neu cells [35] and PC-3 cells [34] show that the lipid composition of these cells is rather similar and cannot explain the different effects of nMase. It would be very interesting to investigate if there is also a high enrichment of ceramide and a low enrichment of SM from cells to exosomes in other cell lines where nSMase affects the release of exosomes. In summary, nSMase and ceramide should not be referred as being important for exosome secretion in general.

The enzyme phospholipase D2 (PLD2) removes part of the head group of phospholipids (mainly PC) producing PA. Thus, this enzyme resembles nSMase since they both reduce the head group size of membrane lipids. Ghossoub et al. showed that PLD2 activity is required for formation of intraluminal vesicles within a fraction of MVBs in MCF-7 cells [82]. In particular, inhibition of PLD2 activity was shown to reduce only the secretion of syntenin-containing exosomes, which make up approximately 50% of the total exosome population in these cells. These results stress the idea that exosomes are not a homogenous group of vesicles, and that exosomes with different composition may require different control mechanisms for their formation and release. Ghossoub et al. performed MS-based lipidomic analyses of MCF-7 cells and their exosomes in both control cells and cells depleted of PLD2 with siRNA. In PLD2-depleted cells the PA level was reduced by approximately 50%, whereas the level of all other lipid classes was not affected [82]. Exosomes released by PLD2-depleted cells had the same level of SM,

PC and PA as the control exosomes, whereas they surprisingly had higher levels of PS, PE and PI. It should be mentioned that PLD2 has earlier been reported to be associated with exosomes, and its activity correlates with the amounts of exosomes released from the mast cell line RBL-2H3 [83]. The mechanism behind this increase is not known, but based on the study in MCF-7 cells [82], it can be hypothesized that PLD2 might be involved in the formation of the intraluminal vesicles in MVBs also in RBL-2H3 cells.

Diacylglycerol kinase α (DGKα) adds phosphate to DAG, thus synthesizing PA. This enzyme has been reported to be present at MVBs and at the plasma membrane of T-lymphocytes, and to have an inhibitory effect on the secretion of exosomes from these cells [84]. It seems as this enzyme reduces the formation of mature MVBs, apparently without affecting the intracellular traffic of MVBs [84]. Thus, studies with PLD2 in mast cells and MCF-7 cells as well as the studies with DGKα in lymphocytes indicate that the levels or formation of DAG and PA are important for the formation and secretion of exosomes. These lipid classes, together with ceramide, have the smallest head groups of the membrane lipids, and point to the size of the head groups as being important for intracellular sorting and trafficking.

So what about a role for other lipid species in exosome release? Addition of the ether lipid precursor HG showed a doubling of ether lipids in PC-3 cells, and revealed that these cells secreted more exosomes than the control cells. The exosomes secreted from cells treated with the ether lipid precursor showed both changes in their lipid content (as discussed above) and their protein composition [43]. Ether lipids have previously been suggested to be involved in membrane fusion [85], making it possible that the effect of adding an ether lipid precursor could be to increase the fusion of the MVBs with the plasma membrane. Since the cells treated with HG contained less MVBs and less intraluminal vesicles per MVB, it was speculated that this was due to cells containing less mature MVBs with fewer intraluminal vesicles due to an increased fusion of MVBs with the plasma membrane [43].

We have recently published that inhibition of formation of $PI(3,5)P_2$ by knockdown of PIKfyve with siRNA or inhibition of this enzyme with apilimod actually increases exosome secretion and seems to inhibit fusion of MVB with lysosomes [25]). Furthermore there are more MVBs with an increased number of intraluminal vesicles in cells with reduced PIKfyve activity. The reported ability of $PI(3,5)P_2$ to bind to and act as an agonist for the lysosomal Ca^{2+} channel TRPML1, may be related to the inhibition of fusion with lysosomes after reduction of $PI(3,5)P_2$ [86].

Of the lipids discussed in this section, we would like to mention that we found only 0.32 mol% of ceramide and 0.16 mol% of PA in exosomes secreted from PC-3 cells (Table 1). We are not aware of any other published data for the mol% of these lipid classes in exosomes. It can be calculated that a lipid bilayer with a diameter of 70 nm contains approximately 60,000 lipid molecules, based on the assumption that each lipid molecule in average occupies 0.4–0.5 nm^2 of the surface area [87]. Thus, there could be approximately 190 ceramide molecules and half of that of PA molecules in such a vesicle. We estimated that PC-3 exosomes contain one protein molecule (sum of membrane-bound proteins and those found inside exosomes) per 65 lipid molecules [34], but the surface area of these proteins is not known. As the plasma membrane of most cells has been estimated to contain approximately 50% of proteins [88], there are probably not more than 100 ceramide and 50 PA molecules per exosome secreted from PC-3 cells.

Although some studies have been published on the importance of lipids and/or lipid-metabolizing enzymes in the formation and release of exosomes, we have to accept that we are still in the very early process of understanding these mechanisms. For future studies and discussions about these issues, it is important to keep in mind that there are cell type dependent regulatory mechanisms for the release of exosomes. Also, there may not only be differences between various cell lines, but even different regulatory mechanisms for different exosome populations within a single cell line, as discussed above for the syntenin-containing exosomes released from MCF-7 cells [83].

7. Clinical applications of exosomes

7.1. Exosomes as vehicles for drug delivery

During the last years there has been an increasing interest in the possibility to use exosomes as vehicles for drug delivery. We refer to recent reviews regarding strategies and the present status of the field [9,11,89–92]. By reading the literature about exosomes as vehicles for drug delivery, one can see that there is much focus on technical issues. Which methods should be used for large-scale isolation of exosomes? How to load exosomes with drugs? Which donor cells to use? Is it possible to use autologous exosomes only or can non-immunogenic exosomes be made? Can targeting molecules be added on the surface? Standardized protocols for purification and analyses are asked for.

The lipid composition of exosomes and their stable membrane structure is certainly an advantage for the stability of such vehicles following intravenous injection. It has even been demonstrated that anthrax toxin can survive in the body for a long time circulating within exosomes [93]. This stability, however, also gives major challenges with the loading of drugs, at least of drugs that are not both very small and hydrophobic. One possibility may be to make exosomes which contain the active constituents (drugs) when being secreted from cells. But still a major hurdle remains in order to make use of exosomes as vehicles for drug delivery. How can it be possible to document the product, including the reproducibility of different batches, as needed to obtain market approval for drugs? Although regulatory issues of exosome-based drug delivery have been mentioned in some reviews [9,89,91], there is not much focus on this challenge in the literature. This should certainly be a main issue to consider for everyone aiming at developing exosomes as drug delivery vehicles. Based on these regulatory approval hurdles, we expect that it will be much easier to benefit from the diagnostic use of exosomes than to use exosomes for drug delivery.

7.2. Exosomes as biomarkers

The diverse content of exosomes makes them an excellent source of noninvasive biomarkers [8,94–97]. During the last years both the protein and nucleic acid content of exosomes has been explored and several candidate biomarkers for a variety of diseases have been identified [8]. The potential use of exosomal lipids as biomarkers has however not been explored in detail.

Due to the high stability of the exosomal membrane one would expect that it will be more challenging to use an immunological-based method like ELISA to measure proteins with their antibody binding site inside exosomes or within the membrane, than to measure such sites present on the outside of the exosomes [98,99]. Thus, it will probably be easier to use methods like Western blots where detergents are used to solubilize the lipid membrane for such analyses, and probably even better to use MS analyses.

This review focuses on exosomal lipids, and some researchers have tried to use lipids in urine as biomarkers [100–102]. In these studies lyophilized urine samples were often used and it was not described how these samples were treated before lyophilization, such that it is unclear if they contain a mixture of cells and vesicles. To our knowledge, the first study of lipid biomarkers in exosomes from urine was performed with samples from 8 patients with renal cell carcinoma and 8 healthy volunteers [100]. The investigators describe their study as a preliminary application of using a hyphenated LC-QTOF-MS platform to analyze lipids in such samples. The signals reported to be different in the two groups were tentatively identified as 35 molecular lipid species. These species were in general very different from what we recently reported for exosomes isolated from urine [40] (see below), and the species giving the largest differences between the two groups in the renal cell carcinoma study was lysoPE 20:4, which was not detect in our urine samples.

As mentioned, we recently reported the lipid composition of exosomes purified from urine of 15 patients with prostate cancer and 13 healthy volunteers [40]. Due to a limited amount of sample from some of these patients, the analyses were performed using exosome preparations containing only 4 μg of protein, and 36 molecular species were quantified (107 species quantified for the data shown in Table 1). Differences were observed for several lipid species in the lipid compositions of the two groups and the highest significance between these groups were observed for the species LacCer d18:1/16:0 (highest in the patient group) and PS 18:1/18:1 (highest in the control group). By combinations of lipid species the two groups were separated with high sensitivity (93%) and specificity (100%). The relative amount of the different lipid classes was, however, too small to discriminate between the two groups, making MS analyses the only method to detect these differences. Thus, these data stress the importance of making MS analyses a routine method in clinical laboratories in order to benefit from lipid analyses of exosomes as biomarkers in the clinic.

Yang et al. [103] very recently published lipidomic data for urinary exosomes collected from four prostate cancer patients and four healthy volunteers. The urine samples were frozen before exosome preparations were prepared and these preparations were then pooled into one cancer sample and one healthy control sample. Their data were presented as the ratio of patients to controls only, i.e. absolute quantification data were not shown. Furthermore, their samples showed a size distribution much larger than expected for exosomes, and these large vesicles were much more abundant in the cancer than the control sample. The large differences in the TAG and CE content of these samples indicate that they may contain not only vesicles larger than exosomes, but also varying amounts of lipid droplets that might be released from intact cells that were frozen prior to vesicle preparation.

8. Summary

We have in this review discussed the current knowledge about the lipid composition of exosomes. Further quantitative data for lipid species in exosomes secreted from different cell lines and tissues are needed to understand the function of exosomal lipids and the variability of the lipid compositions of exosomes. Several studies have shown that different mechanisms can be involved in the formation and secretion of exosomes in different cell lines and also for various types of exosomes secreted from a cell line. There are many similarities between the lipid compositions of exosomes secreted from PC-3 cells and HIV particles. The lipid composition of DRMs, used as models of lipid rafts, also shows some similarities with exosomes, but DRMs deviate more from exosomes than HIV particles. The lipid composition of DRMs strongly indicates that these preparations must contain other structures than the bilayer membrane structures found in exosomes and HIV particles. Finally, analyses of exosomal lipids emerges as a useful approach when looking for biomarkers, although the results available so far indicate that it would be important to make MS analyses a common method in clinical laboratories to fully benefit from this possibility.

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