ELSEVIER

Biochimica et Biophysica Acta 1371 (1998) 351–362

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Docosahexaenoic acid (DHA) alters the structure and composition of membranous vesicles exfoliated from the surface of a murine leukemia cell line

E. Eugene Williams *, Laura J. Jenski, William Stillwell

Department of Biology, Indiana University / Purdue University at Indianapolis, 723 West Michigan Street, Indianapolis, IN 46202, USA

Received 12 December 1997; accepted 2 March 1998

Abstract

Membrane lipid microdomains are regions of the membrane thought to be functionally important, but which have remained poorly characterized because they have proven to be difficult to isolate. The exfoliation of small membranous vesicles from the cell surface is a continuous and normal activity in many cells. If microdomains are relatively large or stable, they may influence the structure and composition of exfoliated vesicles, which are easy to isolate. We tested the ability of docosahexaenoic acid (DHA), a fatty acid proposed to alter the structure of microdomains, to change the structure and composition of vesicles exfoliated from a murine leukemia cell line. Cells were cultured in normal and DHA-enriched media for 72 h, then washed and given a 15-h exfoliation period. Afterwards, the pooled vesicles and their parent plasma membrane were collected and analyzed. Vesicles and plasma membrane from cells grown in normal culture medium had similar fatty acid compositions, including equal, and low, proportions of DHA, but the vesicles had much more cholesterol and displayed higher anisotropy than the plasma membrane. When cells were grown in DHA-enriched medium, both the plasma membrane and exfoliated vesicles had 10-fold elevated levels of DHA in their phospholipids, with the DHA displacing other polyunsaturates. These cells released vesicles having significantly reduced levels of cholesterol and monoenoic fatty acids than those in normal culture. The anisotropy of these vesicles was also dramatically reduced. These data are consistent with DHA altering the structure and composition of membrane microdomains on the cell surface, and suggest that exfoliated vesicles may prove useful in the further study of membrane microdomains.

Keywords: Membrane microdomain; Membrane exfoliation; Membrane structure; Docosahexaenoic acid; Shed vesicle; Lipid microdomain

1. Introduction

The shedding, or exfoliation, of membranous vesicles from the cell surface into the surrounding medium occurs routinely in several types of both normal and transformed cells [1]. The purpose and mechanisms underlying this process are largely unknown. In normal cells, exfoliated vesicles may represent a means of eliminating excess membrane material [2], or be

Abbreviations: DHA, docosahexaenoic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; HPLC, high-performance liquid chromatography; PA-DPH, 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)-phenyl-propionic acid; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acids; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate

* Corresponding author. Fax: +1-317-274-2846; E-mail: ewilliam@iupui.edu

0005-2736/98/$19.00
PII S0005-2736(98)00039-X

involved in extracellular signaling events [3,4]. In transformed cells, these vesicles may be involved in the escape of tumors from immune surveillance [5,6]. Exfoliated vesicles are small (50–200 nm in diameter [4,6,7]), and rich in cholesterol [8,9].

Despite the freedom of lateral movement afforded by the lack of covalent bonds between their constituents, cellular membranes are not homogenous structures. Instead, they are complex, patchy amalgams of membrane domains made up of different lipids and proteins [10–13]. Membrane domains can be divided into two general types: those that arise from lipid–protein interactions and those that arise from lipid–lipid interactions [11,14]. Domains driven by lipid–protein interactions are the more familiar of the two types, and include the basolateral and apical surfaces of polarized epithelial cells [10], and the membrane domains formed by the erythrocyte cytoskeleton [15]. The majority of cellular membrane, however, is believed to be made up of a collection of lipid microdomains formed by the assorted interactions among the many different lipids present in the membrane. The size, life span, organization, and biological significance of these lipid-driven microdomains is unknown [16]. They have so far eluded comprehensive characterization in part because they have proven to be difficult or impossible to isolate. They might be small, shortlived and biologically unimportant structures, or they might be large and stable enough to represent significant functional units of the membrane. If lipid-driven microdomains are relatively large, or numerous, and if they persist in the regions of the plasma membrane that give rise to exfoliated vesicles, then changes in the composition of these microdomains might be discernible as changes in the composition of exfoliated vesicles. Similarly, if an exogenous lipid were to be incorporated into the plasma membrane and then found to alter the content of other lipids within the exfoliated vesicles, this might be interpreted as evidence that microdomains exist on the surface of the living cell.

Docosahexaenoic acid (DHA), an ω-3 fatty acid, is the longest (22 carbons) and most unsaturated (6 C–C double bonds) fatty acid commonly found in the phospholipids of biological membranes. DHA represents a major component of the membranes of only a few, specialized tissues including brain, sperm, and retinal rod outer segments, where DHA can account for up to 50 mol% of the total fatty acids [17,18]. In most tissues, membrane DHA content is much lower, yet can be readily elevated by dietary supplementation [17,18]. When included in the diet or culture medium, DHA is readily taken up by a variety of cells, where it is incorporated into membrane phospholipids [19–21]. Unlike other fatty acids, membrane-associated DHA is tenaciously retained during periods of ω-3 deficiency and starvation [18,22,23]. This defense of membrane DHA content suggests an important cellular role for this molecule, and indeed, DHA-containing phospholipids have a powerful influence on membrane structure. For example, the incorporation of DHA into the plasma membrane of T27A murine leukemia cells increases membrane permeability [24], and alters membrane structure in such a way as to increase the susceptibility of the cells to destruction by the immune system [21]. In model membranes, polyunsaturated fatty acids (PUFA), including DHA, tend to segregate away from regions of cholesterol thereby forming membrane microdomains [25–28], suggesting a role for these lipids in microdomain formation on cellular membranes. Also, DHA is effective at promoting membrane fusion [29]. Since membrane fusion and exfoliation are essentially different manifestations of the same process [30], it is conceivable that DHA has an important effect on vesicle exfoliation. This idea is supported by the fact that those tissues with normally high levels of DHA are specialized for membrane exfoliation and fusion. Therefore, an understanding of how DHA influences exfoliation may provide insight into the organization and structure of the membrane regions that give rise to these vesicles, which in turn will contribute to a better understanding of the processes involved in membrane lipid microdomain formation [31].

Our goal in this work was to compare the lipid composition and molecular order of vesicles exfoliated from the surface of a murine leukemia cell line (T27A) cultured in normal medium and in medium enriched with DHA. The plasma membrane, the originating or ‘parent’ membrane of the exfoliated vesicles, was also isolated and analyzed. Our results verify that DHA is readily incorporated into the plasma membrane of these cells, and that its incorporation leads to alterations in the phospholipid fatty acid profile, cholesterol content, and molecular order

of the exfoliated vesicles. These results are consistent with DHA altering the normal formation of lipid microdomains on the cell surface.

2. Materials and methods

2.1. T27A cell culture

T27A is a myeloid leukemia cell line [32] that continuously exfoliates membranous vesicles. Cells were cultured in the presence and absence of added DHA (see below) in spinner flasks containing RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 15% bovine calf serum (HyClone Laboratories, Logan, UT), 2 mM glutamine, 25 mM HEPES, and antibiotics, in humidified incubation chambers maintained at 37°C with a constant flow of 5% CO_2 in air. Cell density was monitored daily and held between 2×10^5 and 1×10^6 cells/ml. Final cell preparations had been cultured for approximately 72 h (about 6 generations) and contained between 3×10^8 and 4×10^9 total cells.

2.2. Enrichment of culture medium with DHA

DHA was added to RPMI medium at a final concentration of 50 μg/ml using our modification [33] of the method described by Spector and Hoak [17]. The fatty acid (Nu Check Prep, Elysian, MN) in hexane (5 mg/ml) was added dropwise to Celite (Sigma, St. Louis, MO, 1 mg DHA/0.03 g Celite) and then dried in the dark under a gentle stream of nitrogen. The resulting dry DHA/Celite powder was mixed with calf serum-free RPMI prepared as above, but to which 1% fatty acid-free bovine serum albumin had been added. This mixture was stirred for 30 min in the dark, after which the Celite was removed by centrifugation for 30 min at $600 \times g$. The DHA-enriched medium was resterilized by filtration through 0.22 μm filters (Millipore, Bedford, MA). Immediately prior to use, bovine calf serum was added for a total of 15%. The major fatty acids present in both normal and DHA-enriched media, due to the presence of calf serum, were linoleic acid (18:2, 48%), oleic acid (18:1, 22%), palmitic acid (16:0, 10%), and stearic acid (18:0, 6%, all by gas chromatographic analysis, see below). In unmodified medium, DHA made up less than 1% of the total fatty acids.

2.3. Isolation of plasma membranes and exfoliated vesicles

Cells cultured in the absence or presence of supplemental DHA were washed by centrifugation (15 min at $500 \times g$) and then were resuspended in fresh RPMI medium, where vesicle exfoliation was allowed to proceed to 15 h. Exfoliated vesicles (EV) were isolated by centrifugation of culture supernatant for either 45 min at $32\,000 \times g$ (Beckman 42.1 rotor) or for 3 h at $11\,000 \times g$ (Beckman JA-14 rotor) after two initial 15 min spins at $500 \times g$ to remove tumor cells. EVs were subsequently washed by centrifugation in phosphate-buffered saline (PBS, 0.154 M NaCl, 0.016 M NaH_2PO_4, pH 7.2) before being stored at -80°C until further use.

Plasma membranes were isolated using our [20] modification of the method of Kaduce et al. [34] with the buffers described by Molnar et al. [35]. After collection of T27A cells by centrifugation at $500 \times g$ for 15 min, cells were resuspended in 0.25 M sucrose buffer (0.25 M sucrose, 40 mM NaCl, 100 mM KCl, 5 mM $MgSO_4$, 20 mM trizma base, pH 7.2) then disrupted three times by nitrogen cavitation (rapid decompression from 800 psi to atmospheric pressure) in a Parr Instruments pressure vessel. The resulting cellular homogenate was centrifuged at $27\,000 \times g$ for 10 min to remove undisrupted cells and cellular debris, and the supernatant over the pellet was spun for 1 h at $105\,000 \times g$. The resulting pellet was carefully resuspended in a minimal volume of sucrose buffer using a ground glass tissue homogenizer and then spun for an additional 15 min at $580 \times g$ before that supernatant was layered onto a cushion of 1.1 M sucrose solution (other components identical to the 0.25 M sucrose buffer). After a 16-h spin at $107\,000 \times g$ (Beckman SW 27 rotor), purified plasma membranes were removed from the interfacial region and washed free of sucrose by centrifugation for 2 h at $107\,000 \times g$ in excess PBS. Plasma membranes prepared in this way represent a better than 8-fold purification over the crude plasma membrane fraction applied to the one-step sucrose density gradient [34].

2.4. Cholesterol assay

Membrane cholesterol content was determined by quantifying the area of the peak corresponding to

cholestenone (4-cholesten-3-one) eluting from a C-18 high-performance liquid chromatography (HPLC) column after samples of the membrane preparations had been treated with cholesterol oxidase as described by Contreras et al. [36]. Membranes were diluted with PBS containing 50 mM $MgCl_2$, 50 mM tris (pH 7.4), 1 mM dithiothreitol, 0.1% Triton X-100 and 0.5% sodium cholate, and were then incubated for 2 h in the presence of 1 unit of cholesterol oxidase (Sigma). After stopping the reaction with methanol/ethanol (1:1, v/v), and pelleting protein ($13\,000 \times g$, Eppendorf 5415 microcentrifuge), a 50-μl aliquot of the alcohol-stopped reaction mixture was injected onto a 250 mm × 4 mm C-18 column (Beckman, Fullerton, CA) and eluted at 2.0 ml/min with methanol/acetic acid (99:1, v/v). Cholestenone was detected at 240 nm, and peak areas were calculated using Beckman's System Gold Nouveau and compared to the areas of authentic cholestenone standards (Sigma).

2.5. Fatty acid analysis

Membrane lipids were extracted using the chloroform/methanol procedure of Bligh and Dyer [37]. Phospholipids were prepared for gas chromatographic analysis by transesterification to fatty acid methyl esters using methanolic sodium methoxide as described [38]. Fatty acid methyl esters were analyzed using a Shimadzu GC-17A gas chromatograph fitted with a 0.25 mm × 30 m Stabilwax capillary column (Restek, Bellefonte, PA). Fatty acid methyl ester reference standards were provided by Nu Chek Prep. Peak areas were converted to weight of fatty acid using the areas of the standards and Shimadzu's EZ-Chrom software package on a dedicated 486 microcomputer. The phospholipid fatty acid content of each membrane fraction was calculated by summing the individual peak areas.

2.6. Fluorescence anisotropy measurements

The bulk phase molecular order of purified plasma membrane and exfoliated vesicles was assessed after culture in normal and DHA-enriched media. Membrane preparations were diluted with PBS to an absorbance of less than 0.15 AU at 364 nm (to reduce depolarization due to light scattering) before 0.5 μl of a 2-mM solution of the probe of interest in N',N'-dimethylformamide was injected and the mixture stirred in the dark for 30 min. The steady-state fluorescence polarization of the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) and its cationic and anionic derivatives 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate (TMA-DPH) and 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenyl-propionic acid (PA-DPH, all from Molecular Probes, Eugene OR) was measured using a Perkin-Elmer LS 50B luminescence spectrometer with the slit widths set to 5 nm and the sample cell maintained at 37°C with a temperature-controlled, circulating water bath. DPH intercalates deep into the bilayer interior, while TMA-DPH and PA-DPH are thought to partition close to the aqueous interface regions of the exofacial and cytofacial leaflets, respectively [39–43]. Samples were excited at 364 nm and the emission at 430 was recorded with the emission polarizing filter first perpendicular then parallel to the excitation beam. Polarization values (p) were converted to anisotropy (a) by the equation $a = 2p/(3-p)$ [44] after correcting for polarization intrinsic to the monochromaters (G-factor) [45].

2.7. Statistics

The significance of differences between paired means was assessed using Student's *t*-test. When making multiple comparisons ANOVA with the Student–Newman–Keuls post-hoc test was used. Percentage data were arcsine-transformed before statistical analysis as recommended [46]. In all cases, probabilities less than 0.05 were considered significant. Data are presented as means ± 1 S.E.M.

3. Results

High concentrations of DHA are toxic to T27A and other leukemia cells [19,47,48]. Therefore, in these experiments, cells were cultured in medium containing albumin-bound DHA at a concentration (50 μg/ml, 0.152 mM) known to be sufficiently low to allow the cells to survive and proliferate, but that resulted in significant incorporation of the fatty acid into membrane phospholipids (Table 1 and Ref. [19]). In this study, cell viability and growth rates in normal

Table 1
The lipid composition of plasma membrane and exfoliated vesicles collected from T27A murine leukemia cells cultured in normal (−DHA) and DHA-enriched (+DHA) medium

Component	Plasma membrane		Exfoliated vesicles	
	−DHA	+DHA	−DHA	+DHA
DHA	1.08 ± 0.26	13.7 ± 0.53[a]	2.33 ± 0.98	20.7 ± 8.2[a]
Phospholipid fatty acid	109.1 ± 25.3	116.5 ± 37.4	161.2 ± 28.0	156.9 ± 22.8
Cholesterol	10.7 ± 0.47	10.4 ± 0.35	59.2 ± 20.4	20.4 ± 8.6[a]

Values are expressed as mean ng/10^6 cells ± S.E.M. from at least three independent membrane preparations.
[a]Pairs of data that are significantly different.

and DHA-enriched media were identical, confirming that DHA was not present at toxic levels.

The plasma membrane of cells cultured in DHA-enriched medium had more than 10 times the phospholipid DHA content (per cell) as did the plasma membrane of cells grown in normal culture medium (Table 1). This increase was paralleled in the pooled exfoliated vesicles, which had 10 times more phospholipid DHA (also expressed per cell) when they originated from cells grown in DHA-enriched medium than when they were from cells grown in normal medium (Table 1). Although DHA levels increased, the total phospholipid fatty acid content of the two membrane fractions was unaffected (Table 1), suggesting that DHA was incorporated at the expense of other fatty acids. The total cholesterol content of the plasma membrane fractions from the two groups of cells were equal (Table 1). However, the amount of cholesterol recovered in exfoliated vesicles was sharply reduced when the vesicles were formed by cells grown in DHA-enriched medium. The population of vesicles shed from DHA-treated cells had on average less than half the amount of cholesterol found in the vesicles released from cells in normal culture.

The quantitative analysis presented in Table 1 allows direct comparisons to be made only between membranes of the same type because the different procedures used to isolate the two membrane fractions probably had different recovery efficiencies. Since the total amount of fatty acids in each type of membrane was not significantly altered by DHA treatment, the fatty acid content provided a convenient means of standardizing the DHA and cholesterol content of each membrane, so that direct comparisons between the two different membrane types could be made. These standardized data are plotted in Fig. 1. The upper panel shows that when expressed in this way, the plasma membrane of normally cultured leukemia cells and the vesicles exfoliated from them contained equal, and low, proportions of DHA (open bars). When cells were cultured in DHA-enriched medium, both membranes clearly exhibited a pronounced and similar increase in their proportions of

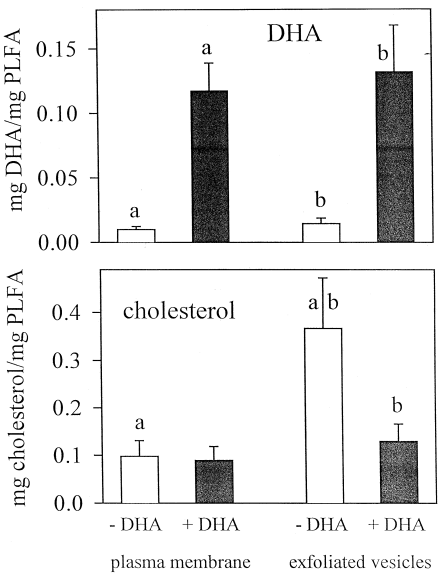

Fig. 1. The DHA and cholesterol content of plasma membrane and exfoliated vesicles from T27A leukemia cells after normal culture (−DHA) and culture in DHA-enriched medium (+DHA). The data have been standardized to the total phospholipid fatty acid (PLFA) content of the separate membrane fractions, and are expressed as means ± S.E.M. of at least three independent membrane preparations. Bars labeled with the same letter are significantly different from one another (statistical analyses were not performed between the comparisons [plasma membrane −DHA/exfoliated vesicles +DHA] and [plasma membrane +DHA/ exfoliated vesicles −DHA]).

DHA (Fig. 1, solid bars). In contrast, the pattern of cholesterol distribution was altered in quite a different manner. The lower panel of Fig. 1 shows that in normally cultured cells, the ratio of cholesterol to total fatty acids was significantly higher in exfoliated vesicles than the plasma membrane from which they originated (open bars). When cells were cultured in DHA-enriched medium, the ratio of cholesterol to total fatty acids in the plasma membrane remained unchanged from its value in normally cultured cells. However, in the vesicles exfoliated from these DHA-loaded cells, the cholesterol-to-fatty acid ratio was dramatically reduced to a level similar to that seen in the plasma membrane (Fig. 1, solid bars). Thus, the accumulated cholesterol content of the two membranes was lower when the cells were cultured in DHA-enriched medium (i.e., the sum of the solid bars in the lower panel of Fig. 1 is less than the sum of the open bars).

The types of fatty acids making up the phospholipid fatty acid pools in plasma membrane and exfoliated vesicles were also substantially affected by the presence of added DHA in the culture medium. When grown in normal culture medium, cells released vesicles having statically similar quantities of all fatty acid groups as the parent membrane (Table 2), although some biologically important differences (e.g., total saturate and PUFA content) may be masked by their variability between cell cultures. After culture in DHA-enriched medium, the increase in DHA content found in the phospholipids of the two membranes was the result of, as expected based on the data presented above, the displacement of other, preexisting fatty acids. DHA dislocated, to some extent, fatty acids belonging to all groups except the saturates (Table 2). However, like the proportions of saturates, the proportion of total polyunsaturates in both membranes remained constant (Table 2). Based on the known distribution of saturates and unsaturates among the *sn*-1 and *sn*-2 position of the major phospholipids [49], this pattern suggests that most of the alterations in membrane composition were occurring in the *sn*-2 position of the phospholipids.

In contrast to the vesicles released by cells in normal culture medium, the vesicles exfoliated from cells after DHA culture had significantly more monoenes, and almost twice the proportions of both dienes and PUFAs other than DHA (i.e., 18:2, 20:2, 22:2, and 20:3$n-6$, 20:3$n-3$, 20:4, 20:5, 22:4, and 22:5) than their parent membrane (Table 2). In addition, when vesicles exfoliated from cells in the two culture media were compared, it was found that culture in DHA − enriched medium resulted in vesicles having, along with a large increase in DHA (and a substantial, though statistically insignificant, decrease in other polyunsaturates), decreased proportions of monoenes. Thus, overall, an increase in the DHA content of the plasma membrane resulted in the exfoliation of vesicles having significantly reduced proportions of monoenes and cholesterol.

Finally, the effects of DHA on the structure of the plasma membrane and exfoliated vesicles were assessed (Fig. 2). The fluorescence anisotropy reported in the membrane interior and near the aqueous interfaces indicated that exfoliated vesicles released from leukemia cells grown in normal culture medium were

Table 2
The percentage fatty acid composition of plasma membrane and exfoliated vesicles from T27A leukemia cells raised in normal (−DHA) and DHA-enriched (+DHA) media

Fatty acid group	Plasma membrane		Exfoliated vesicles	
	−DHA	+DHA	−DHA	+DHA
DHA	1.17 ± 0.49[a]	16.04 ± 1.64[a]	1.28 ± 0.39[b]	11.94 ± 3.11[b]
Saturates	38.99 ± 3.89	38.69 ± 1.02	30.91 ± 3.81	30.42 ± 4.68
Monoenes	39.07 ± 1.53[a]	30.92 ± 1.12[a,c]	38.65 ± 0.70[b]	34.37 ± 0.36[b,c]
Dienes	13.80 ± 3.09	8.85 ± 0.23[a]	20.18 ± 2.67	15.83 ± 1.32[a]
Non-DHA PUFA	19.65 ± 4.81	12.82 ± 0.11[a]	28.77 ± 3.26	23.09 ± 2.98[a]
Total PUFA	20.83 ± 4.47	28.87 ± 2.12	30.05 ± 3.54	35.04 ± 5.08

Values labeled with the same letter are significantly different from one another (statistical analyses were not performed between the comparisons PM and EV + and EV and PM +).
Data are means ± S.E.M. from at least three independent membrane preparations.

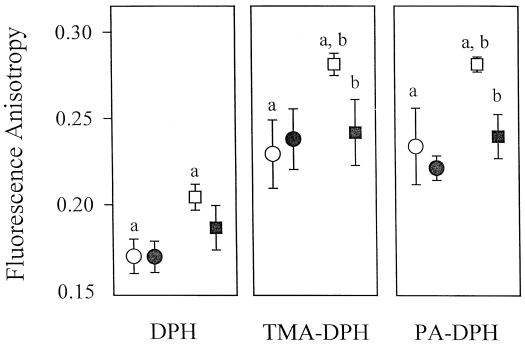

Fig. 2. The fluorescence anisotropy reported by DPH, TMA-DPH, and PA-DPH in the plasma membrane (circles) and exfoliated vesicles (squares) collected from T27A cells after culture in normal (open symbols) and DHA-enriched (filled symbols) media. The data are means ± S.E.M. of preparations from at least three independent membrane preparations. Points labeled with the same letter are significantly different from one another (statistical analyses were not performed between the comparisons [plasma membrane −DHA/exfoliated vesicles +DHA] and [plasma membrane +DHA/ exfoliated vesicles −DHA]).

significantly more ordered (i.e., less fluid) than the plasma membrane which gave rise to them (Fig. 2, open symbols). The culture of the cells in DHA-enriched medium, and the accompanying 10-fold increase in the levels of DHA in the plasma membrane (Table 1), had no effect on the molecular order of that membrane (Fig. 2, circles). However, the large and significant difference in molecular order seen between plasma membrane and exfoliated vesicles from cells in normal culture, disappeared when cells were cultured in DHA-enriched medium (Fig. 2, squares). In DHA-loaded cells, the molecular order of exfoliated vesicles was the same as that their parent membrane.

4. Discussion

The vesicles normally exfoliated from the surface of T27A cells have a statistically similar phospholipid fatty acid composition (Table 2), and a much higher proportion of cholesterol than the plasma membrane from which they arise (Fig. 1). They also exhibit a higher fluorescence anisotropy than their parent plasma membrane in all three membrane regions probed (Fig. 2), indicating a more rigid lipid environment throughout the bilayer. These observations suggest that the composition and structure of the regions of the plasma membrane that ultimately give rise to vesicles are distinct from those of the membrane as a whole. The incorporation of modest, non-toxic amounts of DHA (e.g., 10–15% of total fatty acids), a lipid molecule believed to induce the formation of lipid microdomains in model systems [27], into the plasma membrane significantly alters the phospholipid composition and structure of the vesicles, implying that DHA has a similar domain-inducing effect on the vesicle-forming regions of the cell surface. This is consistent with the idea that a number of regions (microdomains) having distinct lipid compositions exist on the surface of these cells, and suggests that these microdomains are either large enough or numerous enough to significantly influence the properties of sizable areas of the cell surface.

The increased anisotropy of the exfoliated vesicles and their elevated cholesterol content compared to the parent membrane suggest that cholesterol, which has a strong ordering effect on many bilayer lipids (Refs. [25,50], and see below), is a major contributor to membrane order in these vesicles [8,51]. The high cholesterol content of the vesicles released from T27A murine myeloid leukemia cells agrees well with data from vesicles exfoliated from the surface of other leukemia cells [8,9], as well as from several other types of blood cells, all of which shed cholesterol-rich vesicles [3,52,53]. The increased molecular order of exfoliated vesicles compared to that of the plasma membrane is also typical [3,8,9,54].

Differences in the phospholipid fatty acid profile of exfoliated vesicles compared to plasma membrane have also been observed, but these differences appear to be more variable and dependent on cell type or transformation state. For example, it has been reported that the vesicles exfoliated from transformed human lymphocytes have more saturated and less polyunsaturated fatty acids than their originating plasma membrane [3]. Vesicles shed from rat glioma cells have fewer monoenes than the plasma membrane, but more saturated and polyunsaturated fatty acids, including more than twice the amount of DHA [55]. Normal human lymphocytes [3] and another line of murine leukemia cells (GRSL) [8] shed vesicles with a phospholipid fatty acid profile similar to that of those shed by the cells studied here. They have

lower proportions of saturates and higher levels of polyunsaturates than their parent plasma membrane. In addition to differences in cell type and transformation state, diverse methods of lipid analysis and membrane preparation probably contribute greatly to the observed variations.

Culture of T27A cells in DHA-enriched medium results in a substantial increase in the DHA content of the phospholipids in both the plasma membrane and the exfoliated vesicles. Under both normal and DHA-enriched culture conditions, however, the proportion of DHA in the total fatty acid pool of the exfoliated vesicles equals that in the plasma membrane, suggesting that the exfoliation of this fatty acid is directly proportional to its occurrence in the plasma membrane. Although the distribution of DHA in the phospholipid classes has been determined for whole T27A cells [19], it is not known how DHA is distributed among the phospholipid classes or individual molecular species of these particular membranes. The fact that the proportion of saturates was constant while the levels of DHA rose suggests that DHA is paired primarily with 16:0 and/or 18:0, rather than with itself as di-DHA species (e.g., Ref. [56]). It is known that the acyl distribution of the phospholipid classes, including that of DHA, is different in exfoliated vesicles and plasma membrane [55], and it would therefore be interesting to know the detailed distribution of DHA in these membranes.

The sharp rise in the DHA content of the plasma membrane caused by growth in DHA-enriched medium has no effect either on the membrane's cholesterol content or its molecular order. In contrast, growth in DHA-enriched medium has a profound influence on the composition and structure of the exfoliated vesicles; the normally high cholesterol content and molecular order of exfoliated vesicles are both reduced to levels equal to those seen in the plasma membrane (Table 1 and Fig. 2). The fate of the surplus cholesterol (i.e., the cholesterol that fails to be shed when DHA is elevated) is unknown. We did not measure the cholesterol content of other cellular membrane fractions nor the rate of cholesterol synthesis by the cells. It is possible that culture in DHA-enriched medium for 72 h results in reduced cholesterol synthesis or reduced transport of cholesterol to the plasma membrane. Both the feedback control of cholesterol biosynthesis and its transport are known to be altered in many vertebrate tumors [57,58], and it is unclear how the addition of DHA to tumor cell membranes influences this dysfunction. However, the observation that the cholesterol content of the plasma membrane remains constant while that of the vesicles varies, suggests that plasma membrane levels of cholesterol are closely regulated. It is possible that in these cells cholesterol biosynthesis and/or transport to the cell surface is coupled to its exfoliation.

Taken together, the data presented above demonstrate clearly that exogenous DHA is readily incorporated into the phospholipids of the plasma membrane, and that from there it is released into the extracellular medium in exfoliated vesicles in direct proportion to its content in the plasma membrane. Furthermore, the presence of elevated amounts of DHA-containing phospholipids in the plasma membrane causes a reduction in the exfoliation of cholesterol and a concomitant loss of molecular order in the exfoliated vesicles, changes that may reflect those occurring in the vesicle-forming regions of the plasma membrane.

The non-random distribution of phospholipid fatty acids and cholesterol between plasma membrane and exfoliated vesicles in normally cultured cells (Table 2) implies that some directed membrane process is involved in the segregation of certain membrane lipids into specific regions of the membrane. The incorporation of exogenous DHA into the plasma membrane has a strong influence on this process. It has been shown previously in a number of cell types that exfoliated vesicles arise from non-random regions of the cell membrane [1–3,9,59,60]. However, the mechanisms underlying the creation of these vesicles have been elusive (e.g., Ref. [8]). The high molecular order of exfoliated vesicles led to the early suggestion that rigid regions of the membrane are exfoliated, and thus that membrane domains of high molecular order might be involved in vesicle formation [9]. The results of the experiments described here show that vesicles released from cells after culture in DHA-enriched medium have the same low rigidity as the plasma membrane, and thus demonstrate that membrane rigidity per se is not the only membrane property involved in exfoliation. Studies with inhibitors provide evidence that proteins are involved in the process of vesicle exfoliation [55], possibly in the budding and release of the nascent vesicles. How-

ever, the means by which a few specific lipids are made to aggregate into particular regions or domains of the fluid membrane are unclear, and may be separate from those involved in vesicle budding and release. In this case, the role of lipid–lipid interactions within the membrane may be critical.

Many of the lipids of biological membranes do not mix uniformly or ideally. Even lipids of very similar composition tend to interact imperfectly [61,62], and this immiscibility leads to the formation of a large number of dynamic and rapidly changing membrane domains [10,11,16,63]. Two lipid molecules in particular, DHA and cholesterol, appear to have a significant influence on the structure and occurrence of lipid-driven microdomains. For example, cholesterol partitions differently into regions of the membrane having subtle differences in physical properties and saturation states [64]. In addition, cholesterol is able to condense (i.e., decrease the mean area occupied by) phospholipids containing saturated and monounsaturated (monoene) fatty acids [25], but is much less effective on dienes and more polyunsaturated lipids. Cholesterol has no condensing effect on phospholipids containing two unsaturated acyl chains or on phospholipids containing DHA [25,26,28].

These interactions among membrane lipids may be responsible for the non-random distribution of phospholipid fatty acids in the plasma membrane of T27A cells, and might help explain the effects observed here of DHA on the structure and composition of the vesicles released from their surface. Under normal culture conditions, the membrane content of cholesterol and phospholipids with varying degrees of unsaturation are poised such that they contribute to the formation of cholesterol-rich regions that selectively participate in vesicle formation. We speculate that the incorporation of exogenous DHA into the plasma membrane disrupts this balance and causes less cholesterol to be found in those vesicle-forming regions of the membrane. Since cholesterol and DHA-containing phospholipids tend to interact unfavorably [27,28], the increase in DHA content of the plasma membrane may result in the formation of larger than normal DHA-rich microdomains, or induce atypical cholesterol microdomains (e.g., smaller, more fragmented, denser), which may interfere with the usual incorporation of cholesterol and phospholipid molecular species into vesicle-forming regions of the membrane. Another possibility is that a variety of different membrane microdomains give rise to a diverse population of vesicles with different compositions. DHA might play an important role in the formation or maintenance of these microdomains. DHA and other ω-3 fatty acids have recently generated much interest because of their reputed health benefits (e.g., Ref. [65]), including the control of cancer [19,24,66]. How the ω-3's are involved in these effects is unclear, but there is growing evidence that the incorporation of DHA into membrane phospholipids, and its subsequent effects on membrane organization and domain structure, is responsible for a significant portion of its biological effects [19–21,24,66–70]. It is possible that modulation of membrane lipid microdomain structure is a key function of DHA.

5. Conclusion

We have shown that DHA is readily taken up by murine leukemia cells and incorporated into phospholipids of the plasma membrane. Elevated levels of DHA in the plasma membrane have no effect on the molecular order or cholesterol content of that membrane, but are associated with a dramatic reduction in the molecular order and cholesterol content of the membranous vesicles normally exfoliated from the cell surface. These data are consistent with DHA inducing new, or modifying existing, lipid–lipid interactions and thereby establishing unique arrangements of lipid microdomains. Vesicles exfoliated from the plasma membrane, which we have shown to be amenable to manipulation by exogenous fatty acids, and to biochemical and biophysical analysis, might provide useful in future studies of lipid microdomains.

Acknowledgements

This work was supported by NIH Grant #RO1-CA57212 and a grant from Phi Beta Psi.

References

[1] P.H. Black, Shedding from the cell surface of normal and cancer cells, Adv. Cancer Res. 32 (1980) 75–199.
[2] A.R. Beaudoin, G. Grondin, Shedding of vesicular material

from the cell surface of eukaryotic cells: different cellular phenomena, Biochim. Biophys. Acta 1071 (1991) 203–219.
[3] M.J. Armstrong, J. Storch, N. Dainiak, Structurally distinct plasma membrane regions give rise to extracellular membrane vesicles in normal and transformed lymphocytes, Biochim. Biophys. Acta 946 (1988) 106–112.
[4] D.P. Tuck, D.P. Cerretti, A. Hand, A. Guha, S. Sorba, N. Dainiak, Human macrophage colony-stimulating factor is expressed and shed from the cell surface, Blood 84 (1994) 2182–2188.
[5] M.P. Lerner, S.W. Lucid, G.J. Wen, R.E. Norquist, Selected area membrane shedding by tumor cells, Cancer Lett. 20 (1983) 125–130.
[6] V. Dolo, P. Pizzurro, A. Ginestra, M.L. Vittorelli, Inhibitory effects of vesicles shed by human breast carcinoma cells on lymphocyte ^{3}H-thymidine incorporation, are neutralised by anti TGF-β antibodies, J. Submicrosc. Cytol. Pathol. 27 (1995) 535–541.
[7] V. Dolo, E. Adobati, S. Canevari, M.A. Picone, M.L. Vittorelli, Membrane vesicles shed into the extracellular medium by human breast carcinoma cells carry tumor-associated surface antigens, Clin. Exp. Metastasis 13 (1995) 277–286.
[8] W.J. Van Blitterswijk, G. De Veer, J.H. Krol, P. Emmelot, Comparative lipid analysis of purified plasma membranes and shed extracellular membrane vesicles from normal murine thymocytes and leukemic GRSL cells, Biochim. Biophys. Acta 688 (1982) 495–504.
[9] W.J. Van Blitterswijk, P. Emmelot, H.A.M. Hilkmann, J. Hilgers, C.A. Feltkamp, Rigid plasma-membrane-derived vesicles, enriched in tumor-associated surface antigens (MLr), occurring in the ascites fluid of a murine leukemia (GRSL), Int. J. Cancer 23 (1979) 62–70.
[10] M. Glaser, Lipid domains in biological membranes, Curr. Opin. Struct. Biol. 3 (1993) 475–481.
[11] R. Welti, M. Glaser, Lipid domains in model and biological membranes, Chem. Phys. Lipids 73 (1994) 121–137.
[12] T.E. Thompson, M.B. Sankaram, R.L. Biltonen, Biological membrane domains: functional significance, Comments Mol. Cell. Biophys. 8 (1992) 1–15.
[13] M. Edidin, The variety of cell surface membrane domains, Comments Mol. Cell. Biophys. 8 (1992) 73–82.
[14] J.-F. Tocanne, L. Cézanne, A. Lopez, B. Piknova, V. Schram, J.-F. Tournier, M. Welby, Lipid domains and lipid/protein interactions in biological membranes, Chem. Phys. Lipids 73 (1994) 139–158.
[15] W. Rodgers, M. Glaser, Characterization of lipid domains in erythrocyte membranes, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 1364–1368.
[16] M. Edidin, Lipid microdomains in cell surface membranes, Curr. Opin. Struct. Biol. 7 (1997) 528–532.
[17] T. Hendriks, A.A. Klompmakers, F.J.M. Daemen, S.L. Bonting, Biochemical aspects of the visual process: XXXII. Movement of sodium ions through bilayers composed of retinal and rod outer segment lipids, Biochim. Biophys. Acta 433 (1976) 271–281.
[18] N. Salem, Jr., H.-Y. Kim, J.A. Yergey, Docosahexaenoic acid: membrane function and metabolism, in: A.P. Simopoulos, R.R. Kifer, R.E. Martin (Eds.), Health Effects of Polyunsaturated Fatty Acids in Seafoods, Academic Press, Orlando, FL, 1986, pp. 263–317.
[19] M. Zerouga, W. Stillwell, J. Stone, A. Powner, A.C. Dumaual, L.J. Jenski, Phospholipid class as a determinant in docosahexaenoic acid's effect on tumor cell viability, Anticancer Res. 16 (1996) 2863–2868.
[20] L.J. Jenski, L.K. Sturdevant, W.D. Ehringer, W. Stillwell, Omega-3 fatty acid modification of membrane structure and function: I. Dietary manipulation of tumor cell susceptibility to cell- and complement-mediated lysis, Nutr. Cancer 19 (1993) 135–146.
[21] A.W. Pascale, W.D. Ehringer, W. Stillwell, L.K. Sturdevant, L.J. Jenski, Omega-3 fatty acid modification of membrane structure and function: II. Alteration by docosahexaenoic acid of tumor cell sensitivity to immune cytolysis, Nutr. Cancer 19 (1993) 147–157.
[22] R.D. Wiegand, C.A. Koutz, A.M. Stinson, R.E. Anderson, Conservation of docosahexaenoic acid in rod outer segments of rat retina during $n-3$ and $n-6$ fatty acid deficiency, J. Neurochem. 57 (1991) 1690–1699.
[23] A.M. Stinson, R.D. Wiegand, R.E. Anderson, Recycling of docosahexaenoic acid in rat retinas during $n-3$ fatty acid deficiency, J. Lipid Res. 32 (1991) 2009–2017.
[24] W. Stillwell, W.D. Ehringer, L.J. Jenski, Docosahexaenoic acid increases permeability of lipid vesicles and tumor cells, Lipids 28 (1993) 103–108.
[25] R.A. Demel, W.S.M. Geurts van Kessel, L.L.M. Van Deenen, The properties of polyunsaturated lecithins in monolayers and liposomes and the interactions of these lecithins with cholesterol, Biochim. Biophys. Acta 266 (1972) 26–40.
[26] M. Pasenkiewicz-Gierula, W.K. Subczynski, A. Kusumi, Rotational diffusion of a steroid molecule in phosphatidylcholine-cholesterol membranes: fluid-phase microimmiscibility in unsaturated phosphatidylcholine-cholesterol membranes, Biochemistry 29 (1990) 4059–4069.
[27] W. Stillwell, S.R. Wassall, A.C. Dumaual, W.D. Ehringer, C.W. Browning, L.J. Jenski, Use of merocyanine (MC540) in quantifying lipid domains and packing in phospholipid vesicles and tumor cells, Biochim. Biophys. Acta 1146 (1993) 136–144.
[28] W. Stillwell, T. Dallman, A.C. Dumaual, F.T. Crump, L.J. Jenski, Cholesterol versus α-tocopherol: effect on properties of bilayers made from heteroacid phosphatidylcholines, Biochemistry 35 (1996) 13353–13362.
[29] W. Ehringer, D. Belcher, S.R. Wassall, W. Stillwell, A comparison of the effects of linolenic (18:3 omega3) and docosahexaenoic (22:6 omega3) acids on phospholipid bilayers, Chem. Phys. Lipids 54 (1990) 79–88.
[30] P.R. Cullis, B. De Kruijff, Lipid polymorphism and the functional roles of lipids in biological membranes, Biochim. Biophys. Acta 559 (1979) 399–420.
[31] R. Lipowsky, Domain-induced budding of fluid membranes, Biophys. J. 64 (1993) 1133–1138.

[32] B. Chesebro, K. Wehrly, K. Chesebro, J. Portis, Characterization of Ia8 antigen, Thy-1.2 antigen, complement receptors, and virus production in a group of murine virus-induced leukemia cell lines, J. Immunol. 117 (1976) 1267–1274.
[33] L.J. Jenski, G.M. Bowker, M.A. Johnson, W.D. Ehringer, T. Fetterhoff, W. Stillwell, Docosahexaenoic acid-induced alteration of THY-1 and CD8 expression on murine splenocytes, Biochim. Biophys. Acta 1236 (1995) 39–50.
[34] T.L. Kaduce, A.B. Awad, L.J. Fontenelle, A.A. Spector, Effect of fatty acid saturation on α-aminoisobutyric acid transport in Ehrlich ascites cells, J. Biol. Chem. 252 (1977) 6624–6630.
[35] J. Molnar, G. Markovic, H. Chao, Z. Molnar, Glycoproteins of Ehrlich ascites carcinoma cells: separation of plasma and endoplasmic reticular membrane fragments, Arch. Biochem. Biophys. 134 (1969) 524–532.
[36] J.A. Contreras, M. Castro, C. Bocos, E. Herrera, M.A. Lasunción, Combination of an enzymatic method and HPLC for the quantitation of cholesterol in cultured cells, J. Lipid Res. 33 (1992) 931–936.
[37] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[38] K. Eder, A.M. Reichlmayr-Lais, M. Kirchgessner, Studies on the methanolysis of small amounts of purified phospholipids for gas chromatographic analysis of fatty acid methyl esters, J. Chromatogr. 607 (1992) 55–67.
[39] E.E. Williams, J.R. Hazel, Membrane fluidity and hemilayer temperature sensitivity in trout hepatocytes during brief in vitro cold exposure, Am. J. Physiol. 266 (1994) R773–R780.
[40] M.D. Houslay, I. Dipple, L.M. Gordon, Phenobarbital selectively modulates the glucagon-stimulated activity of adenylate cyclase by depressing the lipid phase separation occurring in the outer half of the bilayer of plasma membranes, Biochem. J. 197 (1981) 675–681.
[41] F.G. Pendergast, R.P. Haugland, P.J. Callahan, 1-[4-(Trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene: synthesis, fluorescence properties and use as a fluorescence probe of lipid bilayers, Biochemistry 20 (1981) 7333–7338.
[42] J.-G. Kuhry, P. Fonteneau, G. Duportail, C. Maechling, G. Laustriat, TMA-DPH: a suitable fluorescence polarization probe for specific plasma membrane fluidity studies in intact living cells, Cell Biophys. 5 (1983) 129–140.
[43] S. Kitagawa, M. Matsubayashi, K. Kotani, K. Usui, F. Kametani, Asymmetry of membrane fluidity in the lipid bilayer of blood platelets: fluorescence study with diphenylhexatriene and analogs, J. Mol. Biol. 119 (1991) 221–227.
[44] M. Shinitzky, Y. Barenholz, Fluidity parameters of lipid regions determined by fluorescence polarization, Biochim. Biophys. Acta 515 (1978) 367–394.
[45] J.R. Lakowicz, Principles of fluorescence spectroscopy, Plenum, New York, 1983.
[46] R.R. Sokal, F.J. Rohlf, Biometry, W.H. Freeman, New York, 1981.
[47] L.J. Jenski, L.K. Sturdevant, W.D. Ehringer, W. Pascale, W. Stillwell, Fish oil-rich diets increase tumor cell sensitivity to cell-mediated lysis and alter tumor cell antigen expression, in: R.K. Chandra (Ed.), Nutrition and Immunology, ARTS Biomedical Publishers and Distributors, St John's, Newfoundland, Canada, 1992, pp. 297–307.
[48] C.P. Burns, E.S. Petersen, J.A. North, L.M. Ingraham, Effect of docosahexaenoic acid on rate of differentiation of HL-60 human leukemia, Cancer Res. 49 (1989) 3252–3258.
[49] B.J. Holub, A. Kuksis, Metabolism of molecular species of diacylglycerophospholipids, Adv. Lipid Res. 16 (1978) 1–125.
[50] W. Stillwell, W.D. Ehringer, A.C. Dumaual, S.R. Wassall, Cholesterol condensation of alpha-linolenic and gamma-linolenic acid-containing phosphatidylcholine monolayers and bilayers, Biochim. Biophys. Acta 1214 (1994) 131–136.
[51] W.J. Van Blitterswijk, R.P. Van Hoeven, B.W. van der Meer, Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady-state fluorescence polarization measurements, Biochim. Biophys. Acta 644 (1981) 323–332.
[52] D. Barz, M. Goppelt, M. Szamel, V. Schirrmacher, K. Resch, Characterization of cellular and extracellular plasma membrane vesicles from a non-metastasizing lymphoma (Eb) and its metastasizing variant (ESb), Biochim. Biophys. Acta 814 (1985) 77–84.
[53] T. Araki, Release of cholesterol-enriched microvesicles from human erythrocytes caused by hypertonic saline at low temperatures, FEBS Lett. 97 (1979) 237–240.
[54] W.J. Van Blitterswijk, P. Emmelot, H.A.M. Hilkmann, E.L.P. Oomen-Meulemans, M. Inbar, Differences in lipid fluidity among isolated plasma membranes of normal and leukemic lymphocytes and membranes exfoliated from their cell surface, Biochim. Biophys. Acta 467 (1977) 309–320.
[55] E.G. Trams, C.J. Lauter, N. Salem Jr., U. Heine, Exfoliation of membrane ectoenzymes in the form of micro-vesicles, Biochim. Biophys. Acta 645 (1981) 63–70.
[56] M. Zerouga, L.J. Jenski, W. Stillwell, Comparison of phosphatidylcholines containing one or two docosahexaenoic acyl chains on properties of phospholipid monolayers and bilayers, Biochim. Biophys. Acta 1236 (1995) 266–272.
[57] P.S. Coleman, Membrane cholesterol and tumor bioenergetics, Ann. N.Y. Acad. Sci. 488 (1986) 451–467.
[58] P.S. Coleman, L. Sepp-Lorenzino, The role of the cholesterol synthesis pathway during tumor cell proliferation, in: M. Esfahani, J.B. Swaney, (Eds.), Advances in Cholesterol Research, The Telford Press, Caldwell, NJ, 1990, pp. 201–270.
[59] D.D. Taylor, C.G. Taylor, C.-G. Jiang, P.H. Black, Characterization of plasma membrane shedding from murine melanoma cells, Int. J. Cancer 41 (1988) 629–645.
[60] D.D. Taylor, P.H. Black, Neoplastic and developmental importance of shed plasma membrane fragments, Am. Zool. 26 (1986) 511–514.
[61] A.G. Lee, Lipid phase transitions and phase diagrams: II. Mixtures involving lipids, Biochim. Biophys. Acta 472 (1977) 285–344.

[62] K. Nag, C. Boland, N. Rich, K.M.W. Keough, Epifluorescence microscopic observation of monolayers of dipalmitoylphosphatidylcholine: dependence of domain size on compression rate, Biochim. Biophys. Acta 1068 (1991) 157–160.
[63] M.J. Karnovsky, A.M. Kleinfeld, R.L. Hoover, R.D. Klausner, The concept of lipid domains in membranes, J. Cell Biol. 94 (1982) 1–6.
[64] M.B. Sankaram, T.E. Thompson, Cholesterol-induced fluid-phase immiscibility in membranes, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 8686–8690.
[65] A.P. Simopoulous, R.R. Kifer, R.E. Martin (Eds.), Health Effects of Polyunsaturated Fatty Acids in Seafoods, Academic Press, Orlando, FL, USA, 1986.
[66] L.J. Jenski, M. Zerouga, W. Stillwell, Omega-3 fatty acid containing liposomes in cancer therapy, Proc. Soc. Exp. Biol. Med. 210 (1995) 227–233.
[67] F. Schroeder, A.B. Kier, C.D. Olson, M.E. Dempsey, Correlation of tumor metastasis with sterol carrier protein and plasma membrane sterol levels, Biochem. Biophys. Res. Commun. 124 (1984) 283–289.
[68] L.J. Jenski, K.S. Sturdevant, W.D. Ehringer, W. Stillwell, Omega-3 fatty acids increase spontaneous release of cytosolic components from tumor cells, Lipids 26 (1991) 353–358.
[69] E.A. Dratz, A.J. Deese, The role of docosahexaenoic acid (22:6$n-3$) in biological membranes: examples from photoreceptors and model membrane bilayers, in: A.P. Simopoulos, R.R. Kifer, R.E. Martin (Eds.), Health Effects of Polyunsaturated Fatty Acids in Seafoods, Academic Press, Orlando, FL, 1986, pp. 319–351.
[70] L.J. Jenski, G.M. Bowker, J.M. Scherer, A.C. Dumaual, J. Stone, L. Zhang, W. Stillwell, Modulation of membrane structure and antigen expression by docosahexaenoic acid, FASEB J. 8 (1994) A2321.