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REVIEW

Oxidative Stability of Marine Phospholipids in the Liposomal Form and Their Applications

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Abstract Marine phospholipids (MPL) have attracted a great deal of attention recently as they are considered to have a better bioavailability, a better resistance towards oxidation and a higher content of eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) than oily triglycerides (fish oil) from the same source. Due to their tight intermolecular packing conformation at the *sn*-2 position and their synergism with α-tocopherol present in MPL extracts, they can form stable liposomes which are attractive ingredients for food or feed applications. However, MPL are still susceptible to oxidation as they contain large amounts polyunsaturated fatty acids and application of MPL in food and aquaculture industries is therefore a great challenge for researchers. Hence, knowledge on the oxidative stability of MPL and the behavior of MPL in food and feed systems is an important issue. For this reason, this review was undertaken to provide the industry and academia with an overview of (1) the stability of MPL in different forms and their potential as liposomal material, and (2) the current applications and future prospects of MPL in both food and aquaculture industries with special emphasis on MPL in the liposomal form.

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Abbreviations

AA	Arachidonic acid
BHT	Butylated hydroxytoluene
CHO	Cholesterol
CL	Cardiolipin
DAG	Diacyglycerols
DHA	Docosahexaenoic acid
DP	Diacetyl phosphate
EE	Encapsulation efficiency
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
LA	Linoleic Acid
LPC	Lysophosphatidylcholine
LUV	Large unilamellar vesicles
MLV	Multilamellar vesicles
MPL	Marine phospholipids
n-3 PUFA	Omega-3 polyunsaturated fatty acid(s)
PA	Palmitic acid
PC	Phosphatidylcholine(s)
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL	Phospholipid(s)
PS	Phosphatidylserine
SA	Stearylamine
SPM	Sphingomyelin
TAG	Triacyglycerols
TL	Total lipids
NL	Neutral lipids

Introduction

The present imbalance in the intake of n-3 and n-6 polyunsaturated fatty acids (PUFA) has a serious negative impact on health in the general population [1–3] and there is a strong desire to improve the situation by introducing new products on the market with a higher level of n-3 PUFA and a lower level of n-6 PUFA. Currently, the global food and dietary supplement market for n-3 fatty acids (EPA and DHA) is estimated to be 15,000–20,000 tons, derived from a total world production of fish oil of approximately 300,000 tons per year. Marine phospholipids (MPL) from, e.g., krill represents an alternative source of n-3 PUFA, but the market for MPL is still in its infancy even though an increasing activity in this field has been observed recently [4]. A number of companies are preparing market introduction of either natural MPL, derivatives of natural MPL, or synthetic MPL. The leading MPL product on the market at the moment is a krill extract with approximately 35% PL [5]. There are also MPL products that are made from fish processing by-products and salmon roe. It is expected that the MPL market will follow the general trends of n-3 fish oils. MPL are new on the market and their range of applications has yet to be determined. However, MPL are believed to have potential applications in human and animal nutrition, in pharmacology, and in drug delivery. The most well-documented applications of MPL are related to liposomes. Liposomes made from MPL have been developed as a test system for antioxidants and as model systems for oxidation of biological membranes [6–9].

Many studies have been performed on n-3 triacyglycerols (TAG) enriched functional foods [10] while limited studies have been carried out on MPL enriched functional foods either in their pure form or in liposomal form. Furthermore, the current applications of phospholipid liposomes are limited to lecithin from soy bean or phosphatidylcholine (PC) from egg yolk and no attempts to use MPL based liposomes for food purposes have been reported in the literature [11–13]. However, some studies [14–19] have investigated the use of MPL such as herring roe or krill PL for larvae feed in the aquaculture industry. The limited application of MPL and liposomes in both food and aquaculture industries can be attributed to several reasons (1) lack of knowledge especially related to the behavior of MPL in food and feed systems, (2) limitations in large scale production of liposomes without using organic solvents and (3) the requirement of expensive equipment for liposome production. Nevertheless, there is ongoing research in this area [20–28]. With the growing understanding of the following areas regarding (1) the physicochemical properties of MPL, (2) the oxidative stability of MPL or MPL based liposomes under gastrointestinal condition and (3) emerging technologies for liposome production without using organic solvents such as microfluidization and pro-liposomes method [29], it may soon become feasible to use MPL in both the food and aquaculture industries. This review gives an overview of our current knowledge on the above mentioned aspects.

Classification and Sources of MPL

PL can be divided into three classes: glycerophospholipids, ether glycerolipids and sphingophospholipids. Glycerophospholipids represent the most widespread phospholipid class and they differ in their polar head groups. For example, phosphatidylcholine (PC) has choline as a head group, while phosphatidylethanolamine (PE) has ethanolamine as a head group, etc. as shown in Fig. 1. In addition, PL from different sources also have different fatty acid profiles in the *sn*-1 and *sn*-2 positions (Fig. 2a). Thereby, the chain length and degree of unsaturation may vary from source to source. For example, PL originating from plants such as soy bean do not have fatty acid chain lengths longer than 18 carbon atoms and contain only one to three double

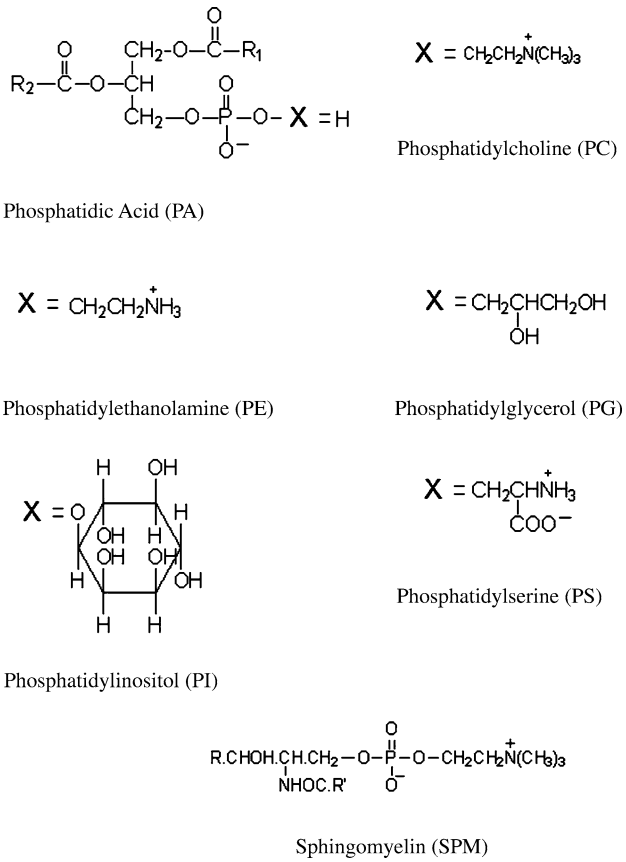

Fig. 1 Chemical structures of PL compounds with names and abbreviations

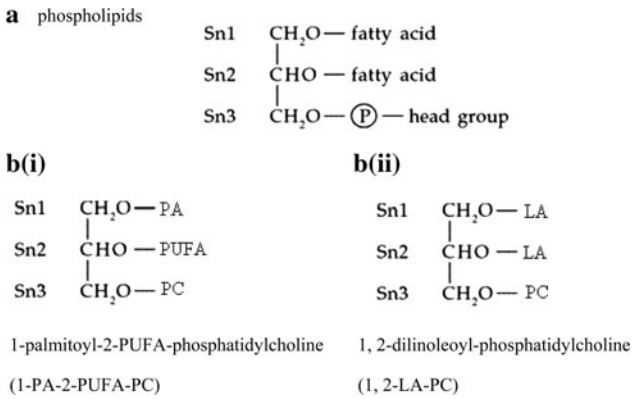

Fig. 2 **a** General structure of a phospholipid, **b** i) 1-palmitoyl-2-PUFA-phosphatidylcholine ii) 1,2-dilinoleoyl-phosphatidylcholine.

bonds, while PL originating from egg yolk or marine sources additionally have chain lengths of 20 and 22 carbon atoms with four to six double bonds e.g. as found in fatty acids of EPA and DHA. However, egg yolk only contains small amounts of EPA and DHA while marine sources are high in EPA and DHA. As far as marine sources are concerned, PL are found relatively abundant in roe, fish heads and offal such as viscera [30]. The most predominant PL in marine source such as salmon, tuna, rainbow trout and blue mackerel is phosphatidycholine (PC) as shown in Table 1. The second most abundant is phosphatidylethanolamine (PE). Phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SPM) and lysophosphatidylcholine (LPC) are usually found in smaller amounts in marine sources, except for the relatively high level of sphingomyelin (SPM) found in tuna species [31–36]. Furthermore, krill such as *Euphausia superba* and *Euphausia pacifica* are other rich source of MPL [37, 38]. Almost half the lipid content of both types of krill is present in phospholipid form, mainly around 35% PC and 16% PE in *Euphausia superba* and 29% PC and 26% PE in *Euphausia pacifica*, respectively. Currently, Neptune Krill oil (a concentrate of MPL from *Euphausia superba*) is a leading commercial krill oil on the market.

Similar to the production of egg yolk PL, production of MPL in industry uses a combination of organic solvents such as hexane and acetone, isopropanol and ethanol for extraction of wet or dried biomass [36]. Non-polar solvents are used to extract TAG while polar solvents are used to extract PL. However, extraction of lipids using organic solvents may bring adverse health effects. Recently, a more promising method without using an organic solvent, supercritical fluid extraction (SFE) has been used for the extraction and fractionation of lipids [39–42]. The extraction can be carried out at low temperature by using CO_2. However, CO_2 can only extract neutral lipids from lipid mixtures, and a generally recognized as safe (GRAS) co-solvent such as ethanol must also be used to extract PL for the food industry. For instance, the addition of about 5–10% of ethanol to CO_2 is necessary to achieve the extraction of PL from egg yolk [42–44]. Additionally, krill oil has been extracted by a patented cold vacuum extraction process that can protect the biomass from exposure to heat, light or oxygen. Thereby, the oil is protected throughout the production process and the original nutrients of the krill are maintained intact.

Health Benefits of MPL

Many studies have shown that MPL are more efficient carriers of n-3 PUFA than TAG (normal fish oils) in terms of n-3 PUFA absorption in different tissues [45–47]. Thus, MPL not only contains more n-3 PUFA than TAG from the same source [31, 48, 49], but also provide better absorption in most tissues. This may be due to the amphiphilic properties of PL resulting in better water dispersability and

Table 1 Phospholipid composition (%) of marine sources

PL classes	Salmon head lipids	Rainbow trout fillet lipids	Bigeye muscle lipids	Bluefin muscle lipids	Bonito muscle lipids	Frigate muscle lipids	Skipjack muscle lipids	Yellowfin muscle lipids	Krill	Salmon roe
PC	54.7	53.6	42.1	42.2	53.9	47.4	51.5	37.9	87.5	86.0
PE	14.0	22.9	18.8	18.9	20.1	21.8	20.2	21.0	6.3	6.0
PI	2.5	8.3	5.8	6.7	2.3	10.9	4.9	8.5	0.5	2.0
PS	10.4	4.1	5.4	4.8	2.2	5.1	5.0	5.4	0.5	ND
SPM	8.3	4.9	3.3	5.6	7.6	3.0	0.5	4.0	1.3	2.0
LPC	1.4	ND	22.1	15.4	13.8	12.0	18.3	21.5	ND	2.0
Cardiolipin	ND	6.2	ND	ND	ND	ND	ND	ND	ND	ND
Other	ND	ND	4.4	6.6	Trace	1.7	1.5	2.8	3.9	1.0

Data compiled from references [5, 31–36]

PC phosphatidylcholine, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PS* phosphatidylserine, *SPM* sphingomyelin, *LPC* lysophosphatidylcholine, *ND* not determined

their greater reactivity towards phospholipases compared to the glycerolysis of triglycerides [49]. For this reason, supplementation of foods with n-3 PUFA rich PL has recently emerged as an interesting way of increasing the assimilation and thereby the health benefits of EPA and DHA. EPA and DHA have numerous well-documented health benefits, which have been reviewed extensively by Narayan et al. [50]. The more recent studies on these health benefits include a reduction of coronary heart diseases, inflammation, autoimmune diseases, hypertension, cancer, diabetes, susceptibility to mental illness and neurological diseases such as depression and Alzheimer's disease, as well as improved brain and eye functions in infants [51–59].

Apart from the benefits obtained from their favorable fatty acid composition, MPL may also provide health benefits due to their polar head groups [60, 61] or to a unique combination of the two in the same molecules. The latter explanation is supported by the following observations; the use of n-3 fatty acids (EPA and DHA) in PL form (either from marine or synthetic origin), instead of the triglyceride form, together with a vegetable oil containing n-6 fatty acids in a nutritive lipid emulsion, gave even lower blood triglyceride and cholesterol levels of patients as compared to the same amount of n-3 fatty acids given as fish oil [62]. The same observation was also obtained by Bunea et al. [63] who investigated the effect of krill oil (mainly present as PL) on hyperlipidemia. In addition, they reported that high doses of krill oil significantly reduced low-density lipoproteins (LDL) level and increased high-density lipoproteins (HDL). Their study concluded that krill oil was more effective at improving blood lipids and lipoproteins than fish oil. Apart from that, several studies have also shown that krill oil has many beneficial health effects such as it may has therapeutic value for metabolic syndrome, non-alcoholic fatty liver disease, attention deficit/hyperactivity deficit disorder (AD/HD), premenstrual syndrome (PMS) and it also showed anti-inflammatory effect [64–68]. Sampalis et al. [67] reported that phospholipid krill oil was more effective than triglyceride fish oil at improving both the physical and emotional symptoms of PMS while Deutsch [66] reported that the intake of krill oil at a daily dose of 300 mg can significantly inhibit inflammation and reduce arthritic symptoms within a short treatment period of 7 and 14 days. According to Maki et al. [64], 4 weeks of krill oil supplementation increased plasma EPA and DHA of overweight and obese men and women and was well tolerated without adverse effects on safety parameters. Besides that, Hayashi et al. [69] also showed that n-3 PUFA from salmon roe phosphatidylcholine may be beneficial in treatment of chronic liver diseases while Taylor et al. [70] showed that MPL is a promising new dietary approach to tumor-associated weight loss. Due to these numerous health benefits, there is an increasing desire to offer MPL containing n-3 PUFA to a wider market, e.g. for human foods and also to the general feed and aquaculture industry.

Introduction to Liposomes

Liposomes or lipid vesicles are aggregates formed from aqueous dispersions of amphiphylic molecules such as polar lipids that tend to produce bilayer structures [71]. They are useful microscopic carriers for nutrients and have a great potential for applications in both food and aquaculture industries. Besides that, liposomes have been recognized as a powerful tool in the treatment of diseases by the pharmaceutical industry. Their use as drug delivery vesicles and their medical applications such as in anticancer therapy, vaccination, gene therapy, and diagnostics have been reported in literature [72]. According to Watwe et al. [73], liposomes can be divided into three main classes: (a) multilamellar vesicles (MLV), contain more than a single bilayer membrane with a size range of 0.1–6.0 μm, (b) small unilamellar vesicles (SUV) and (c) large unilamellar vesicles (LUV) which both contain only a single bilayer membrane with sizes range of 0.02–0.05 μm and >0.06 μm, respectively. LUV are the most useful liposomes because they are more homogeneous than MLV and have higher encapsulation efficiency [74]. MPL or MPL based liposomes have obtained considerable attention and their oxidative stability has been studied extensively as shown in Table 2. Generally, MPL have been found to have a higher oxidative stability than TAG as will be discussed in the following.

Oxidative Stability of MPL

Mechanism of Oxidation for MPL

The PUFA chains in PL are the primary targets of oxidation. Similar to the oxidation of TAG, phospholipid oxidation may occur through radical and non-radical reactions involving enzymes such as lipoxygenase and myeloperoxidase or non-enzymatic systems such as ·OOH, ·OH, Fe^{2+}, Cu^{+} and radiation [75]. Due to the low dissociation energy of bisallylic carbon–hydrogen in double bonds of PUFA, a hydrogen atom can easily be removed. The first steps in the lipid peroxidation consist of hydrogen abstraction, rearrangement of double bonds and addition of triplet oxygen leading to highly reactive peroxyl radicals. These radicals can undergo a large variety of consecutive reactions including further reaction with other PL, fragmentation and generation of truncated PL and different

Table 2 Chemical and physical stability of MPL and MPL-based liposomes

Sources of phospholipids (PL)	Brief summary of findings	References
TL, NL and PL (from muscle of blue fish)	Antioxidant activity in salmon oil system supplemented with: 2.5% or 5% PL > 0.02% BHT 5% PL > 5% TL or 5% NL	King et al. [87]
Lipid fractions (from muscle, viscera and skin of sardine and mackerel fish)	Oxidative stability of lipid fractions: Muscle > viscera and skin Presence of higher PL (PE and PC) and α-Toc in muscle and synergistic effect of PE with α-Toc	Ohshima et al. [105]
Salmon roe PC, soybean PC	Oxidative stability of both PC in aqueous solution 1) Catalyzed by Fe^{2+}-ascorbic acid; salmon roe PC > soybean PC 2) Under influence of emulsifier: egg albumin > Tween 20 > deoxycholic acid sodium salt Reason: high stability of salmon roe PC is due to the conformation of PC molecule and the phase behavior of PC aggregation	Miyashita et al. [90]
Squid: muscle TL, viscera TL, eye TL; Tuna orbital TL, trout egg TL and bonito TAG	Oxidative stability of lipids fraction: Squid viscera TL or squid muscle TL > squid eye TL > trout egg TL > bonito TAG > tuna orbital TL Reason: higher stability is due to the presence of PL in squid tissue lipids and trout egg TL	Cho et al. [21]
DHA, PC, PE, TG	Oxidative stability of DHA in lipids: 1-DHA-2-palmitoyl-PE or 1-palmitoyl-2-DHA-PE or 1-DHA-2-palmitoyl-PC or 1-palmitoyl-2-DHA-PC > DHA + 1,2-palmitoyl-PC (1:1) > 1,2-diDHA-PC + 1,2-dipalmitoyl-PC (1: 1) or 1,2,3-triDHA-TAG DHA was most protected against oxidation when it was incorporated at one position of either PC or PE	Lyberg et al. [9]
Fish roes: salmon and herring, commercial fish oils: crude tuna oil and sardine oil	Oxidative stability of lipids Herring roe lipids > salmon roe lipids > commercial fish oils The higher oxidative stability is mainly due to the presence of PL in fish roe lipids and the synergistic effect of PL on the antioxidant activity of α-tocopherol	Moriya et al. [25]
Salmon roe PC, chicken egg PC and commercial soybean PC	Oxidative stability of PC in: a) Aqueous micelles: Salmon roe PC > chicken egg PC > soybean PC b) Liposomes: Chicken egg PC and salmon roe PC > soybean PC Reason: Higher stability is due to the presence of PUFAs in chicken egg PC and salmon roe PC which are esterified at the *sn*-2 position	Nara et al. [6]
Salmon roe PC, chicken egg PC and commercial soybean PC	Oxidative stability of liposomes containing DHA enriched TAG: Salmon roe PC > chicken egg PC and commercial soybean PC Addition of CHO; DP, SA, chicken egg albumin and Toc improved oxidative stability of salmon roe PC liposomes	Nara et al. [7]
68% PC, 23% PE, 2% PI, 2% PS and 1% SPM, 27% CHO and 4% TAG	Low pH led to an instantaneous vesicle aggregation of MPL-liposomes and shortened the release time of vitamin B1	Cansell et al. [20]
68% PC, 23% PE, 14% EPA, 31% DHA	MPL-liposomes exhibited relative high membrane physical and chemical stability in the gastric digestion condition indicating that MPL-liposomes could be used as oral administration vectors	Nacka et al. [28]
68% PC, 23% PE, 2% PI, 2% PS and 1% SPM	Acidification caused liposomes size and shape changes while maintaining the bilayer structure indicating that MPL-liposomes could be used as oral administration vectors	Nacka et al. [27]
68% PC, 23% PE, 14% EPA, 31% DHA	α-Toc uptake after oral delivery: MPL liposomes > sardine oil digestion Under gastrointestinal condition, α-Toc incorporation improved chemical stability of liposome suspension with best oxidative stability at (5 mol%)	Nacka et al. [26]

Table 2 continued

Sources of phospholipids (PL)	Brief summary of findings	References
Cod roe PL	Lipids oxidation is proportional to [Fe^{2+}] and [PL] but was dependent on pH with a maximum between pH 4 and 5 Addition of salt decreased the rate of lipid oxidation	Mozuraityte et al. [22]
Cod roe PL	Cations did not influence the rate of oxidation in ionic strength 0–0.14 M. Phosphate was more effective in reducing the oxidation rate than chloride. Salts and pH affected the zeta potential of the liposomes	Mozuraityte et al. [23]

TL total lipids, *NL* neutral lipids, *PL* phospholipids, *PC* phosphatidylcholine, *TAG* triacyglycerols, *PE* phosphatidylethanolamine, *CHO* cholesterol, *DP* diacetyl phosphate, *SA* stearylamine, *TOC* tocopherol

types of low molecular weight compounds such as aldehydes and ketones. However, enzymatic oxidation of PL can be eliminated in the MPL during their thermal production. Besides that, different PL oxidation products can be formed depending on the predominating oxidative process [76]. Oxidation products can be classified into three main categories such as: (1) long chain products that preserve the PL skeleton, and which may result from insertion of oxygen followed by rearrangement or cleavage of the PL hydroperoxides leading to epoxy, polyhydroxy, hydroxy, or keto derivatives of PL, (2) short-chain or truncated products, formed by cleavage of the unsaturated fatty acids. These products include ketones, aldehydes, unsaturated carboxylic acids, (keto)hydroxyl-aldehydes, (keto)hydroxyl-carboxylic acids, lyso-phospholipids and lyso-phospholipid halohydrins, and (3) adducts, formed by reaction between oxidation products and molecules containing nucleophilic groups, this include the products usually formed by cross-linking reactions between PL oxidation products with carbonyl groups and amino groups present in neighboring biomolecules such as peptides, proteins and phosphatidylethanolamine.

Dangers of Auto-Oxidation of MPL

Oxidation of MPL can not only deteriorate the quality of MPL enriched foods and affect the flavor, but also promote the development of neurodegenerative diseases. Many reported studies [75, 77–83] have shown that oxidized PL cause harmful effects to human health as they play physiopathological roles in developing diseases such as age-related and chronic diseases, acute lung injury, atherosclerosis, inflammation and decrease immune response. PL oxidation products such as hydroperoxyl, hydroxyl, aldehyde and epoxy groups that are potentially important in the progression of atherosclerosis and inflammation [80]. For instance, by activating the receptor for the platelet-activating factor (PAF), oxidized PL induce platelet aggregation [84–86]. Oxidized PL can also induce monocyte adhesion to endothelial cells, accumulate in atherosclerotic lesions, and play a role in inflammation and signaling inflammatory response. The dangers of the oxidized PL have been reviewed extensively and will not be further discussed in this review.

Antioxidant Effect of PL

King et al. [87] investigated the role of PL and the degree of fatty acid unsaturation on lipid oxidation in a salmon oil model system. Their findings showed that addition of a 2.5% (wt/wt) or a 5% (wt/wt) PL fraction extracted from bluefish to salmon oil increased its stability during heating at 55 and 180 °C as compared to the control salmon oil, or salmon oil to which 0.02% (wt/wt) of BHT or 5% (wt/wt) of other lipid fractions from bluefish such as total lipid or neutral lipid had been added. The PL fraction with 34% DHA was found to exhibit higher oxidative stability than other lipid fractions with 15% DHA. Subsequently, they investigated the antioxidant properties of individual PL in a salmon oil model system [88]. They found that nitrogen-containing PL such as PE, PC, LPC, and SPM were equally effective as antioxidants and they were more effective than PS, PG and PI. Their studies did not postulate any mechanism or reasons for the antioxidant properties of the different PL classes. In both studies by King and colleagues, the oxidative stability of the salmon oil model system was investigated through 2-thiobarbituric acids (TBARS) assay and the decreases in the ratio of DHA to PA (C22:6/C16:0). Boyd et al. [89] investigated the effect of 0.5% (by weight) PL toward lipid oxidation of 2.5 g salmon oil and menhaden oil model systems respectively, through the more sensitive headspace gas chromatographic analysis. Their study also showed that addition of PL significantly reduced the production of volatile compounds in both oil model systems.

Conformations of PUFA at the *sn*-2 Position of PL

Miyashita et al. [90] showed that salmon roe PC had a higher oxidative stability than soybean PC in an aqueous solution dispersed with chicken egg albumin although the degree of unsaturation in the salmon roe PC was higher

than in the soybean PC. They suggested that the high stability of salmon roe PC was mainly correlated with the conformation of the PC molecule and the phase behavior of PC aggregation. The main molecular species of soybean PC was 1,2-dilinoleoyl-phosphatidylcholine (1,2-diLA-PC), while for salmon roe PC it was 1-palmitoyl-2-PUFA-phosphatidylcholine (1-PA-2-PUFA-PC) as shown in Fig. 2b. Hence, the presence of this main molecular species in salmon roe PC (with most of the PUFA located at the *sn*-2 position of PC) may provide a more tightly packed molecular conformation as compared to the soybean PC and thereby increase resistance of PC towards oxidation. The findings of Miyashita et al. [90] corroborated the original work of Applegate and Glomset [91] who reported that DHA in the *sn*-2 position of diacylglycerol (DAG) containing a saturated acyl chain in the *sn*-1 position could form a tighter intermolecular packing conformation as will be further discussed below.

Conformations of DHA at the *sn*-2 Position in a DAG Model

Applegate and Glomset (1986) used a molecular modeling approach to search for conformations of DHA that might uniquely influence acyl chain packing in cell membranes. Their DHA conformations of lowest energy as shown in Fig. 3 were extended conformations in which six double bonds projected outward from the methylene axis (a) in two nearly perpendicular planes to form an extend angle-iron shaped structure or (b) at nearly 90° intervals to form a helical structure, respectively. Studies of packed arrays of these hexaenes with or without saturated hydrocarbons showed that tight packing arrangements were possible especially for angle iron-shaped molecules as a consequence of back-to-back, intermolecular contacts involving these chains. Applegate and Glomset [92, 93] further concluded that different unsaturated fatty acids at the *sn*-2 position of *sn*-1,2-diacylglycerols (DAG) may promote different packing and conformations. For instance, 1-stearoyl-2-DHA-DAG and 1-stearoyl-2-AA-DAG can assume a regular shape and tight packing while 1-stearoyl-2-oleoyl-DAG adopt a highly irregular shape and much looser packing. The simulations by Applegate and Glomset were done without reference to potential effects of polar headgroups, water of hydration and applied thermal energy. However, the molecular areas obtained for the model of DAG are in good agreement with that of the *sn*-2 polyunsaturated phosphoglycerides [94, 95]. This raises the possibility that corresponding natural phosphoglycerides may be able to pack closely together in monolayers and bilayers if their headgroups do not interfere. The findings of Applegate and Glomset were supported by Albrand et al. [96] who also agreed with the existence of the extended-helical conformations of DHA in PL. However, they also suggested several coiled conformations for DHA, tightly back-folded helical conformations with 1.2 and 1.5 spirals appearing to be the most stable as shown in Fig. 3.

More Recent Studies on the Conformation of PUFA at the *sn*-2 Position of PL

Nara et al. [6, 7] further compared the oxidative stability of PC from salmon roe, soybean and chicken egg in aqueous micelles and also in the form of liposomes with and without encapsulation of lipophilic substances. In aqueous

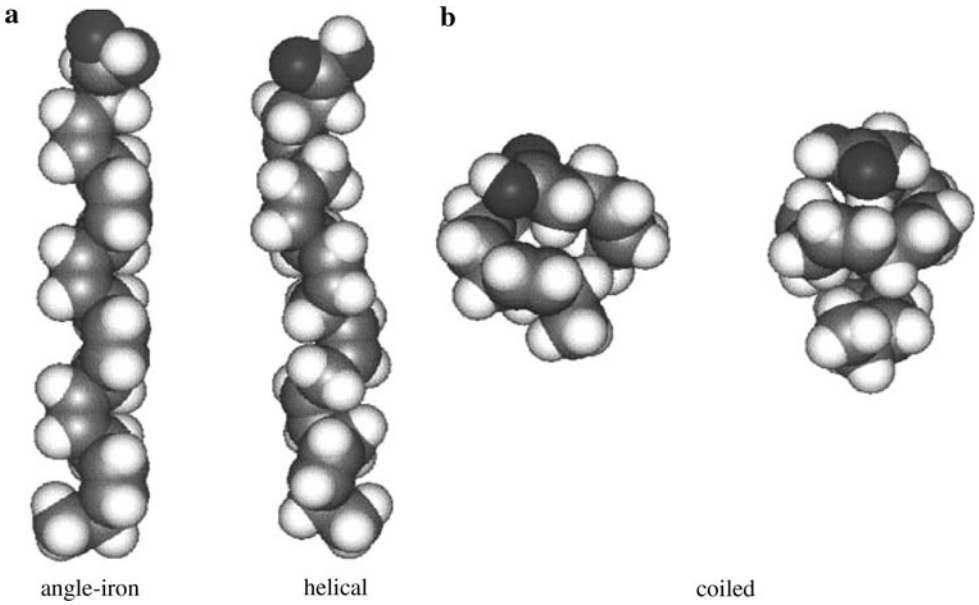

Fig. 3 Extended conformations of DHA in (**a**) angle-iron shaped and helical form, (**b**) coiled form

micelles, salmon roe PC was found to have the highest oxidative stability as evaluated by the highest content of un-oxidized PUFA, followed by chicken egg PC and soybean PC. Their findings are in agreement with the findings of Miyashita et al. [90]. No significant difference was found in oxidative stability between chicken egg PC and salmon roe PC when in the pure form of liposomes. However, for liposomes encapsulating with DHA enriched TAG resulted in the highest oxidative stability of both TAG and PC when salmon roe PC was used as the encapsulation material [7]. This unusual order of oxidative stability could be expected to be closely related to the conformation of PUFA at the *sn*-2 position in PC molecules as mentioned earlier [91]. Consequently, it is difficult for free radicals and oxygen to attack PUFA in bilayers of tighter conformation in salmon roe PC liposomes. Nara et al. [7] also suggested the possibility of using salmon egg PC as a liposomal material for the prevention of the oxidation of encapsulated fish oils.

Furthermore, Araseki et al. [8] also reported the characteristic oxidative stability of PC liposomes prepared from synthesized PC containing palmitic acid (PA), linoleic acid (LA), arachidonic acid (AA) and docosahexaenoic acid (DHA) in known positions. When the oxidative stability of 1-PA-2-LA-PC or 1-PA-2-AA-PC was compared with that of a 1:1 (mol ratio) mixture of 1,2-diPA-PC + 1,2-diLA-PC, or 1,2-diPA-PC + 1,2-diAA-PC respectively, the PC were more oxidatively stable than the latter corresponding PC mixtures in all oxidation systems despite the fact that the degree of unsaturation was the same in 1-PA-2-PUFA-PC and the corresponding mixture of PC. This was suggested to be due to the different conformation of PC bilayers which refer to the location of PUFA at the *sn*-2 position and the different rate of hydrogen abstraction by free radicals from intermolecular and intramolecular acyl groups. Their finding did not support a study by Lyberg et al. [9] who reported that the stability of DHA was improved independent of its position (*sn*-1 or *sn*-2) in PC or PE. Besides that, the more recent experiments and simulations [97–102] emphasized various degrees of flexibility of the DHA chain that gives looser packing of lipids bilayer. Their NMR analysis showed that the mobility of the hydrophobic part of the DHA molecule is higher than that of LA in liposome formation. These two competing views were portrayed in a review by Gawrish et al. [103]. However, according to Saiz and Klein [100], the flexibility of DHA chain conformation gives looser packing of the membrane at the lipid water interface and causes high water permeability. The presence of water molecules near DHA molecules lowers the density of the bisallylic hydrogen and inhibits the hydrogen abstraction from double bonds of PUFA during the propagation stage of autoxidation. As a conclusion, the higher water permeability of DHA and its specific conformation may be a reason for higher oxidative stability of DHA or other PUFA containing liposomes.

However, as compared to the study mentioned earlier by Miyashita et al. [90], contradictory results have also been reported by Monroig et al. [15, 16, 19] in their efforts to develop PUFA-rich liposomes for fish feed. They found that liposomes made from krill PL with 67% PC, 9% PE and a high content of PUFA showed lower oxidative stability as compared to liposomes made from soybean lecithin with 95% PC. The contradictory findings may be due to the different experimental conditions in the two studies, liposomes in model system versus liposomes in *Artemia* enrichment condition. In the model system, liposomes were formulated with pure PC containing fatty acid chains in known positions of the glycerol moiety and the oxidation was carried out in a very well-defined condition (temperature of 37 °C, in the dark and without agitation). On the contrary, the *Artemia* enrichment conditions were as follows: enrichment was carried out at 28 °C with strong aeration and 21 h of incubation.

Synergism Between PL and α-Tocopherol

Many studies have shown that the higher stability of PL may be due to the presence of antioxidants such as α-tocopherol in the PL mixture or synergistic effects of PL together with α-tocopherol [21, 25, 87, 88, 104–107]. The mechanism responsible for the synergy of tocopherols and PL is not very well understood. However, Hildebrand et al. [108] postulated that the mechanism involved in synergism of PE, PC and PI with tocopherol in the autoxidation of soybean oils were as follows: (1) amino groups of organic bases in PE and PC molecules and reducing sugar in the PI molecule facilitate hydrogen or electron donation to tocopherol and (2) these PL extend the antioxidant efficacy of tocopherol by delaying the irreversible oxidation of tocopherol to tocopherylquinone. Additionally, Saito et al. [106] reported that antioxidant activity of PL was found to be attributable not only to side chain amino groups such as choline and ethanolamine, but also to the hydroxyl group in the side chain.

Oshima et al. [105] studied the oxidative stability of sardine and mackerel lipids with respect to synergism between phospholipids and α-tocopherol. They investigated the oxidative stability of lipid fractions from different parts of sardine and mackerel; tissue from white and red muscles, viscera and skin of the fish. The oxidative stability was determined through the measured changes of the peroxide value (PV), fatty acid composition, α-tocopherol content and the oxygen uptake of lipids during an incubation period at 37 °C. Muscle lipids, which contain α-tocopherol and larger amounts of PL (PE and PC) than

other tissues, showed good oxidative stability despite their high content of PUFA. It was postulated that the synergistic effect of PE with α-tocopherol was the main reason for this phenomenon. Cho et al. [21] compared the oxidative stability of lipid fractions from marine organisms, squid muscle total lipids (TL), squid viscera TL, squid eye TL, tuna orbital TL, trout egg TL and bonito TAG. The fatty acid compositions, lipid classes, tocopherol contents and average number of bisallylic positions in each lipid fraction are shown in Table 3. Higher oxidative stabilities of three kinds of squid tissue TL and trout egg TL compared to those of bonito TAG and tuna orbital TL were observed as shown in Fig. 4. The authors suggested that the presence of PL in lipid fractions from squid tissue and trout egg was responsible for this increased oxidative stability. In addition, bonito TAG was found to be less susceptible to oxidation than tuna orbital TL and this could be due to the presence of a higher tocopherol content in bonito TAG.

Moriya et al. [25] compared the oxidative stability of fish roe lipids (salmon roe and herring roe) with that of lipids from commercial fish oils (crude tuna oil and crude sardine oil). As shown in Table 4, fish roe lipids contain higher levels of PL, EPA and DHA, and lower levels of tocopherol while lipids from commercial fish oils contain higher levels of TAG, tocopherol and lower EPA and DHA levels. Judging from these data, fish roe lipids were presumed to have lower oxidative stability. However, the opposite was observed as shown in Fig. 5 and it was proposed that the higher oxidative stability of fish roe lipids was mainly due to their high content of PL. It was also suggested that the synergistic effect of PL on the antioxidant activity of tocopherol was the main reason for this phenomenon. The higher oxidative stability of herring roe as compared to salmon roe was suggested to be due to synergism between PE and tocopherol. As shown in Table 4, the PE content in herring roe lipids was 6.6%, but there was no PE in salmon roe. Furthermore, herring roe also contained higher levels of PS and lysoPC than salmon roe and this may also have caused differences in their oxidative stability. The presence of antioxidants other than tocopherols in fish roe lipids such

Table 3 Composition of lipids from marine sources

Fatty acids (wt%)	Squid muscle TL	Squid viscera TL	Squid eye TL	Tuna orbital TL	Trout egg TL	Bonito TAG
14:0	2.1	4.4	0.9	2.9	3.6	3.3
16:0	32.7	15.9	23.2	17.0	10.7	16.3
18:0	4.4	2.9	5.6	3.0	3.0	4.1
18:1n-7	1.3	3.1	1.6	2.9	3.3	2.4
18:1n-9	1.3	8.7	0.2	23.8	15.8	13.8
20:1n-7	ND	2.8	ND	ND	1.7	ND
20:1n-9	2.5	4.2	3.4	1.8	1.8	0.9
18:2n-6	0.2	1.3	1.4	ND	1.1	3.6
18:3n-3	0.1	ND	0.2	ND	1.5	ND
20:3n-3	ND	ND	4.8	0.5	2.7	ND
20:4n-6	1.9	1.7	ND	2.0	0.7	ND
20:5n-3	10.6	12.3	15.1	4.8	18.4	0.6
22:6n-3	38.1	22.5	37.7	21.0	19.8	26.1
No. of bisallylic positions[a]	2.51	2.11	2.77	1.65	2.19	1.92
Lipid class (% of total lipids)						
Triacyglycerols	ND	95.5	ND	99.3	76.8	99.6
Free fatty acids	ND	ND	ND	0.4	ND	0.1
Glycolipids	ND	ND	6.8	ND	ND	ND
Sterols	23.7	0.7	28.3	ND	2.2	0.3
Phospholipids	75.6	3.8	66.4	0.2	23.1	ND
Tocopherol content (μg g^{-1} lipid)						
α-tocopherol	649.8	212.5	1198.8	541.3	215.5	253.4
β-tocopherol	ND	ND	ND	ND	ND	193.3
γ-tocopherol	ND	ND	ND	ND	ND	703.6
δ-tocopherol	ND	ND	9.2	ND	9.2	496.3
Total tocopherol	649.8	212.5	1208.0	541.3	215.5	1646.6

Data from reference [21]

ND not detected

[a] Per one fatty acid molecule

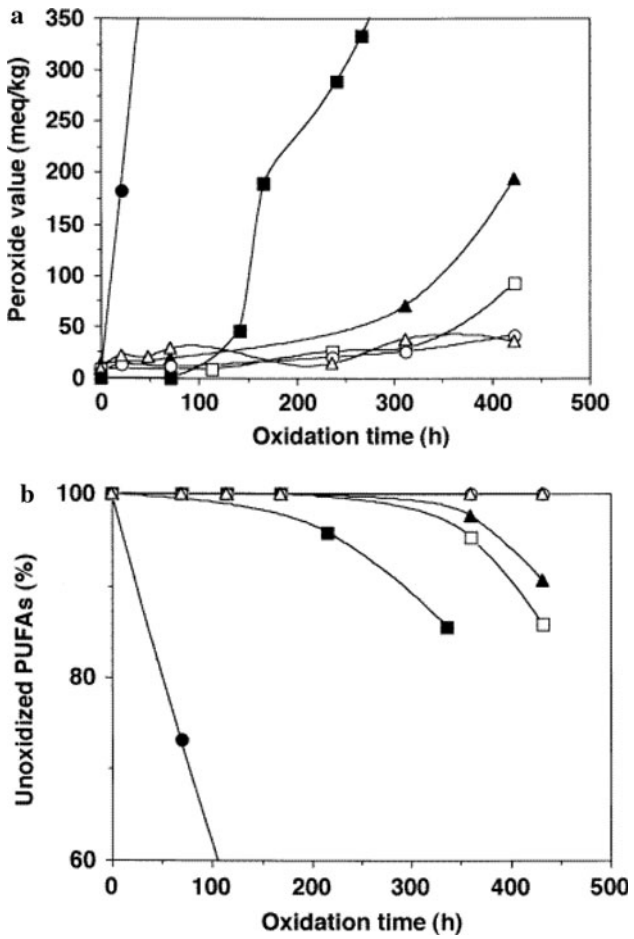

Fig. 4 **a** Changes in the peroxide value (PV) and **b** unoxidized PUFA in lipids from marine organisms during auto-oxidation at 37 °C. (*open triangle*) Squid viscera total lipids (TL); (*open circle*) squid muscle TL; (*open square*) squid eye TL; (*filled circle*) tuna orbital TL; (*filled triangle*) trout egg TL; (*filled square*) bonito oil. Reproduced from Cho et al. [18] with permission from John Wiley & Sons Ltd

as astaxanthin, coenzyme Q10 and lutein might contribute to this extraordinary stability as well.

Other studies [104, 107, 109] reported that the synergistic effect of PE with α-tocopherol was higher than that of PC. Bandarra et al. [104] investigated the antioxidant synergy of α-tocopherol (0.04%) with several PL fractions (0.5%) such as PE, PC and cardiolipin (CL) in a refined sardine oil model system. Their results showed that PC was the most effective individual antioxidant when it was compared to PE, CL and α-tocopherol while PE provided the highest synergistic effect with α-tocopherol. Higher synergism of PE as compared with that of PC could be due to the easier hydrogen transfer from the amino group of PE to tocopheroxyl radical and regeneration of tocopherol or the secondary antioxidant action of PE in reducing quinones formed during oxidation of tocopherols [109]. Since MPL may contribute to better oxidative stability than marine TAG, it can be expected that enrichment of foods or food emulsions with MPL could lead to n-3 PUFA enriched foods that have better oxidative stability than foods enriched with n-3 TAG.

Stability of MPL Based Liposomes Under Gastrointestinal Conditions

MPL based liposomes were designed with the purpose of increasing the PUFA bioavailability and also to protect entrapped compounds from digestive degradation. However, liposome characterization with respect to vesicle composition and membrane integrity under various gastrointestinal conditions are needed before considering liposomes as a useful oral dosage form. Many studies have shown that MPL liposomes could be used as an oral administration vector [6, 7, 20, 26–28]. This is because bilayer structures of MPL based liposomes were still maintained even under acid stress or gastrointestinal conditions despite of slight morphological modifications. Nacka et al. [28] investigated the in vitro behavior of MPL based liposomes under the influence of pH from 1.5–2.5 (stomach) to 7.4 (intestine) at physiological temperature (37 °C) in the presence of bile salts and phospholipase A_2 (Table 2). Their study showed that acidification induced instantaneous vesicle aggregation of MPL based-liposomes, which was partially reversed when the external medium was neutralized. Acidification also caused a complex morphological bilayer rearrangement and led to the formation of small aggregates. Nevertheless, Nacka et al. [27, 28] reported that the pH and temperature dependent structural rearrangement is mainly due to the osmotic shock and chemical lipid alterations such as oxidation and hydrolysis. Hydrolysis of the liposomes was amplified under the influence of an acid medium and high temperatures (Table 2).

Cansell et al. [20] investigated the physical stability of MPL-based liposomes containing vitamin B1 under acidic conditions simulating the stomach conditions. Encapsulation of vitamin B1 in the liposomes was carried out through passive encapsulation and active loading methods. They observed that vitamin B1 was totally released from liposomes after 24 h storage in a neutral medium and the time of release was shortened to 1 h in acidic condition (pH 1.5). According to their study, this liposome instability could result from the external medium osmolarity that forced water to flow out of the liposomes and simultaneously dragged vitamin B1 molecules through the bilayer. Furthermore, protons may also destabilize the lipid membrane by their interaction with PL via structural membrane rearrangement as previously mentioned. However, their study also proved that addition of xanthan gum improved the encapsulation efficiency and also the retention of vitamin B1 in liposomes regardless of the encapsulation

Table 4 Composition of marine lipids used for oxidation

Lipid class (% of total lipids)	Crude tuna oil	Crude sardine oil	Salmon roe	Herring roe
Triacyglycerols	99.6	99.8	71.8	9.3
Free fatty acids	0.1	0.2	ND	3.8
Phospholipids	ND	ND	23.1	73.6
Sterols + monoacylglycerols	0.3	ND	7.2	12.3
% of phospholipids				
PC	ND	ND	97.0	72.3
PE	ND	ND	ND	6.6
PS	ND	ND	2.6	8.7
LysoPC	ND	ND	ND	11.8
Fatty acid profiles				
14:0	3.3	4.1	3.6	2.1
16:0	16.3	8.0	10.7	25.8
18:0	4.1	1.4	3.0	2.2
18:1n-7	2.4	2.0	3.3	5.1
18:1n-9	13.8	10.9	15.8	13.2
20:4n-6	–	1.3	0.7	1.0
20:5n-3 (EPA)	0.6	21.8	18.4	14.4
22:6n-3 (DHA)	26.1	13.7	19.8	21.6
EPA + DHA	26.7	35.5	38.2	36.0
Tocopherol content (μg g^{-1} lipid)				
α-tocopherol	253.4	60.2	19.6	22.9
β-tocopherol	193.3	45.7	214.1	258.0
γ-tocopherol	703.6	376.7	11.6	7.7
δ-tocopherol	496.3	2670.9	11.3	11.5
Total tocopherol	1472.6	3153.5	256.6	300.1
Other antioxidants (μg g^{-1} lipid)				
Astaxanthin	ND	ND	156	ND
Coenzyme Q10	ND	ND	24	100
Lutein	ND	ND	ND	6.4

Data from reference [25]

ND not determined, *LysoPC* lysophosphatidylcholine, *PE* phosphatidylethanolamine, *PL* phospholipids, *PS* phosphatidylserine

method used. They suggested that this increase is due to the adsorption of hydrocolloid to the outer surface of the liposomes that not only trapped part of the external vitamin but also formed a strong xanthan gum coating around the liposome surface. They postulated that this coating resulted from strong lipid–hydrocolloid interactions occurring during the centrifugation steps of liposome preparation.

The Effect of Lamellarity, pH, Temperature, Ionic Strength, Presence of Pro-oxidants and Chelators on MPL-Based Liposomes' Stability

Chemical and physical stability of liposomes are closely related to the mechanical strength and lipid bilayer conformation. Strong and well-packed lipid bilayers or multilamellar layers can protect the entrapped substance, decrease the changes of size distribution, fusion or other changes in the mechanical properties of lipid bilayers. For this reason, factors such as lamellarity, pH, temperature, ionic strength, dissolved oxygen content within the formulation, the presence of antioxidants and chelators are believed to affect mechanical properties of lipid bilayers and thereby affect the physical and chemical stability of MPL-based liposomal products [22, 23].

Nacka et al. [27] showed that the sensitivity of MPL based liposomes towards harsh condition such as acidic condition depends on their size and lamellarity (Table 2). They found that filtered liposomes with higher lamellarity and a protective effect against aggregation showed a slower size rearrangement. This finding supported a study by Monroig et al. [19] who, in addition, reported that liposomes with multilamellar vesicles seem to be more suitable than liposomes with unilamellar vesicles in the encapsulation of free methionine. They found that methionine dissolved in the more internal intermembrane spaces of multilamellar liposomes would remain encapsulated, whereas methionine from the aqueous compartments located between the more outer membranes would leak out

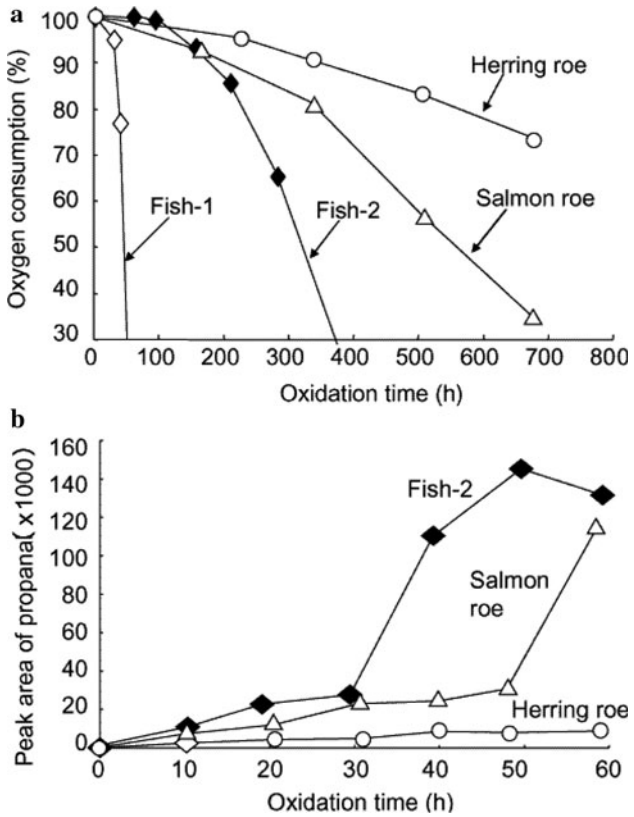

Fig. 5 **a** Oxygen consumption during the oxidation of fish lipids at 37 °C in the dark. (*open diamond*) fish-1; (*filled diamond*) fish-2; (*open triangle*) salmon roe lipids; (*open circle*) herring roe lipids. **b** Propanal formation during the oxidation of fish lipids at 37 °C in the dark. (*filled diamond*) fish-2; (*open triangle*) salmon roe lipids; (*open circle*) herring roe lipids. Reproduced from Moriya et al. [22] with permission from John Wiley & Sons Ltd.

into the external medium when the liposomes were subjected to harsh conditions. However, this result contradicts another study by this group [15] where unilamellar liposomes were found to be more stable than multilamellar liposomes. The apparent discrepancy in these two studies is probably due to different experimental conditions and materials used for the liposomes preparation.

Mozuraityte et al. [22] examined the lipid oxidation rate of liposomes made from cod PL under influence of factors such as the temperature, the amount of added Fe^{2+}, the lipid concentration, pH, the concentration of NaCl, and the dissolved oxygen. Their study showed that the rate of lipid oxidation was proportional to the iron and lipid concentrations. Furthermore, lipid oxidation was dependent on pH, with a maximum observed between pH 4 and 5. Addition of NaCl decreased the rate of lipid oxidation. However, contradictory results were reported in another study [110] which showed that addition of NaCl had no effect or even increased iron-catalyzed oxidation of a sodium dodecyl sulfate-stabilized salmon oil emulsion.

Mozuraityte et al. [23] examined the effect of zeta potential on the lipid oxidation rate of liposomes made from cod PL under the influence of pH and different cations such as Na^+, K^+, Ca^+, Mg^+ and anions such as $H_2PO_4^-$ and Cl^- (Table 2). Their data showed that cations did not influence the rate of oxidation in the tested range of the ionic strength from 0 to 0.14 M whereas the opposite was the case for anions. Both phosphate and chlorides have an additive antioxidative effect on the oxidation in liposomes. Phosphate was shown to be more effective in reducing the oxidation rate than chloride. The inhibition of Fe^{2+} induced oxidation of liposomes by phosphate might be due to the phosphate chelation of iron [111, 112]. Furthermore, they also concluded that addition of salts and changes in pH affected the zeta potential of the liposomes. However, absolute values of the zeta potential alone cannot be used to predict oxidation rates.

Improvement of MPL Based Liposomes' Oxidative Stability

Many studies have been conducted to improve the oxidative stability of liposomes. Most of the studies focus on the use of cholesterol in improving the oxidative stability of liposomes [12, 113–115]. For example, a study conducted by Nara et al. [7] showed that addition of cholesterol and ingredients such as diacetyl phosphate (DP) and stearylamine (SA) improved the oxidative stability of salmon roe PC liposomes. Furthermore, in the effort of developing liposomes as feed supplement in larva culture. Monroig et al. [15] also showed that addition of cholesterol to liposomes made from krill PL or 1,2-PA-PC or soy PC improved the oxidative stability of the liposomes. Cholesterol has a condensing effect on the PC bilayer arrangement over its phase transition temperature and thus improves the physical stabilization of PC liposomes [116]. Addition of cholesterol can increase the rigidity of 'fluid state' liposomal bilayers and the retention of entrapped hydrophilic substances [117]. It counteracts lipids phase transition and increases resistance to in vivo liposomes degradation [118–120]. An interaction mechanism between bilayer forming PL and cholesterol has been proposed. This is due to the formation of hydrogen bonds between the three hydroxyl group of cholesterol and fatty acyl esters of PL at both *sn*-1 and *sn*-2 positions [121, 122]. These physico-chemical effects of cholesterol on liposomes may contribute to the increased oxidative stability in liposomes with cholesterol.

α-Tocopherol is widely known for its antioxidative effect [123]. However addition of high concentrations of α-tocopherol may also cause prooxidative effects [124, 125]. The most effective concentration of α-tocopherol in the prevention of lipid oxidation in salmon roe PC liposome

suspensions was 0.25 μM in a study conducted by Nara et al. [7]. Nacka et al. [26] investigated the most efficient amount of α-tocopherol for liposomes incorporation under gastrointestinal-like conditions. Their findings showed that the best oxidative stability was obtained for liposomes that were prepared at a ratio of 5 mol% of α-tocopherol of the total marine lipids. This concentration of α-tocopherol produced liposomes with the lowest concentration of propanal as an oxidation product of n-3 PUFA and required the longest time of oxidation induction phase. They also found that incorporation of α-tocopherol induced liposome structural modifications, evidenced by turbidity and the production of lysophospholipids from PL chemical hydrolysis.

Nara et al. [6, 7] investigated the effect of addition of diacetyl phosphate (DP), stearylamine (SA) and chicken egg albumin, and soybean protein on improving the oxidative stability of MPL based-liposomes. DP and SA give a negative or positive charge to the liposomes respectively and thus protect the liposomes from aggregation. An improved oxidative stability of liposomes after addition of this ingredient was observed and suggested that it was due to the physical stabilization of the PC liposomes. Furthermore, added proteins such as chicken egg albumin and soybean protein improved the oxidative stability of liposomes by protecting the PC bilayer from the attack of free radicals. Proteins have the ability to absorb at PC–water interfaces and this adsorption of proteins would closely relate to its antioxidant activity [6]. However, albumin acted as a more effective inhibitor of the oxidation of PC containing DHA than PC containing LA [90].

Determination of Oxidation Products from MPL

As discussed above MPL has been found to exert antioxidative effects toward lipids oxidation. However, many of the lipid oxidation studies [6–8, 21, 90, 105] were performed using simple analyses such as TBARS, PV, determination of the un-oxidized lipids (PUFAs) content through gas chromatography, or determination of only one secondary volatile compound, propanal (as a marker of n-3 PUFA oxidation) by headspace GC–MS analysis [25], etc. In many of these oxidative stability studies, there is a lack of determination of the entire spectrum of volatile oxidation products or identification of specific oxidation products which are responsible for sensory off-flavors of the marine lipids. Furthermore, there are no studies providing the sensory data or statistical correlation between instrumental analysis and sensory data for oxidation of MPL. These data are particularly important in the studies of MPL for foods enrichment and additional studies in this area are clearly needed. Due to the low odor threshold, the presence of volatile secondary oxidation products, even at low concentrations, can significantly decrease the sensory quality of marine lipids or marine lipids containing foods.

In the recent years, the oxidation products of PL have attracted intensive research interest due to their biological functions in human pathophysiology. Similar to other lipids such as TAG, many methods can be used to study the oxidation of PUFA containing PL such as (1) measurement of lipid hydroperoxides through spectrophotometric determination of PV or conjugated dienes (CD). Lipid hydroperoxides may also be determined by sample derivatization followed by HPLC with chemiluminescence detection, (2) measurement of breakdown products of hydroperoxides, such as the aldehydes, malondialdehyde, etc. through anisidine value (AV), 2-thiobarbituric acid value (TBARS), etc., (3) measurement of secondary volatile compounds through more sensitive instrumental methods such as GC–MS, (4) measurement of long chain oxidation derivatives of PL through MS. Electrospray ionization (ESI) is gaining in popularity in this area nowadays for this purpose [76]. ESI is a soft ionization technique that does not cause fragmentation and allows detection of intact PL classes without sample derivatization. ESI can readily be coupled to reverse phase LC and allow the analysis of oxidized PL [126–129]. Interfacing reverse phase LC to ESI–MS has the advantage as oxidized PL elutes earlier than their native counterparts due to their higher hydrophilicity. Spickett et al. [127] used the positive ion ESI–MS for detection of hydroperoxide in PC vesicles after treatment with *tert*-butylhydroperoxide and Fe^{2+} while Yin et al. [129] used ultra performance liquid chromatography (UPLC) coupled with negative ion electrospray ion trap MS to identify the intact oxidation products of glycerophospholipids in vitro and in vivo such as hydroxyeicosatetraenoates (HETE) and isoprostanes (IsoP). Other soft ionization methods include matrix-assisted laser desorption ionization (MALDI) and tandem mass spectrometry (MS/MS). As a conclusion, the future direction for research and development could focus on the investigation of oxidative stability for MPL by using advanced MS analysis.

Potential of MPL as Liposomal Material

A variety of liposome preparation methods are available nowadays ranging from traditional methods using solvent extraction such as thin film hydration, detergent dialysis, reverse-phase evaporation, etc. to emerging technologies without using an organic solvent such as pro-liposome, supercritical fluid extraction, and microfluidization. Each method has its own advantages and drawbacks as reviewed by Taylor et al. [130]. Among these technologies, pro-liposome and microfluidization are recommended to produce liposomes for food applications. Pro-liposome is a

simple method for mass production of liposomes without using large amounts of energy, solvents and complex equipment. This method is based on the idea that addition of water to an appropriate mixture of ingredients leads to the spontaneous formation of liposomes [29]. On the other hand, microfluidization is a method using a microfluidizer (a high pressure homogenizer) that can rapidly produce large volumes of liposomes in a continuous and reproducible manner. The average size of the liposomes can be adjusted through this technology and the solutes to be encapsulated are not exposed to sonication, detergents or organic solvents. Furthermore, this technology enables the production of stable liposomes with high encapsulation efficiency [74]. Recently, Thompson et al. [131–133] used a microfluidization technique to produce liposomes from milk fat globule membrane PL in the food industry. Studies showed that liposomes prepared via microfluidization have high encapsulation efficiencies, smaller size, a narrower size distribution and a higher proportion of unilamellar vesicles as compared to methods such as thin film hydration. PL from soybean and egg yolk, either in purified form, crude form or hydrogenated form are widely used for liposome production in both the food and aquaculture industries. The use of MPL-based liposomes has gained attention recently in the aquaculture industry and there is much ongoing research in this area as shown in Table 5. Several studies have shown the use of MPL such as herring roe or krill PL for larvae feed in the aquaculture industry [14–19] but no attempts to use MPL based liposomes for food purposes have been reported in the literature so far.

One potential advantage of using MPL-based liposomes for food application is that they may provide better bioavailability of encapsulated nutrients [26, 134, 135] as compared to TAG. Nacka et al. [26] showed that MPL-based liposomes facilitated α-tocopherol uptake after oral delivery as compared to sardine oil digestion. Furthermore, Hossain et al. [136] also showed that MPL-based PC liposomes (squid PC and starfish PC) enhanced the permeability, transportation and uptake of PL in Caco-2-cells. It is also known that the fluidity of liposomes increases with increasing contents of highly unsaturated PUFA such as AA and DHA, showing the advantage of PC containing AA or DHA for use in drug or nutrient delivery systems [100, 101].

Application of PL Liposomes in the Food Industry

The uses of liposomes in the food industry can be summarized as follows (1) use of liposomes to encapsulate food ingredients in order to provide better protection or to hide the bitter taste of entrapped substances and (2) use of liposomes to control the delivery of functional components by delaying the release of the encapsulated materials. Liposomes have been used to entrap thermally sensitive compounds such as vitamins, enzymes, flavorings, PUFA from fish oils, antimicrobial peptides (lysozyme, nisin) and other nutrients [13, 137–144]. Hydrophilic substances can be entrapped in the internal water core of the liposomes while lipophilic compounds can be efficiently enclosed in the PL bilayer at the same time through a pro-liposomes approach [29]. For this reason, liposomes can be used for the formulation of functional foods or drinks such as energy drinks, sport drinks, fortified milk, etc. Arnaud et al. [145] reported that PC from egg or soybean has been used in development of liposome-based functional drinks. With the use of PC-based liposomes in food industry, consumers not only benefit from the health benefits of water soluble nutrients that are entrapped in the liposomes but also benefit from the nutritional benefits of PL in liposomes. In the production of cheese, PL liposomes may be used to delay the release of encapsulated proteinases [146, 147] or to protect encapsulated enzyme such as protease and lipases with the purpose of improving the texture and sensory properties of cheese [148–152]. Liposomes have also been used to encapsulate vitamin D with the purpose of increasing the vitamin D content of cheese [153].

Application of PL Liposomes in the Aquaculture Industry

Besides food incorporation, recent studies have also indicated that liposomes rich in n-3 PUFA can offer a range of benefits when used for fish larvae feed. Due to the high consumer demand and limited natural stocks of fish species such as salmon, trout and eel, much effort has recently been spent by researchers on developing cost effective aquaculture methods for farming such species. Generally, the main problems faced by aquaculture industry are low survival rate of the hatched fish larvae of the farmed species and the difficulty in supplying live prey organisms which provide nutritionally adequate feed for these larvae. Live prey such as Rotifers *Brachionus plicatilis* and *Artemia* nauplii provide adequate amounts of protein and energy. However, they do not provide lipid profiles that cover the requirements for EPA and DHA, which are essential for optimum survival, growth and development of larvae [154–157]. Thus, to provide prey organisms with such a composition of n-3 PUFA, it is necessary to cultivate these organisms in the presence of enrichment products with high EPA and DHA contents, preferably in an easily digestible, highly bio available form, such as MPL. During the enrichment process, enrichment products are passively filtered by *Artemia* nauplii and their digestive tract becomes loaded with these enrichment products. A wide

Table 5 Application of liposomes in the aquaculture industry

Sources of liposomes	Brief summary of findings			References
Purified PC, CHO, PG, menhaden oil	It is feasible to use liposomes for *Artemia* nauplii enrichment with PL and free amino acids such as glycine, liposomes were readily ingested and assimilated by *Artemia* nauplii as indicated by ^{14}C-glycine and ^{14}C-PC			Ozkizilcik and Chu [169]
1,2-PA-PC, egg PC, bovine brain PS	It is feasible to use liposomes in aquaculture as a delivery system through *Artemia* nauplii. PUFA rich liposomes were stable at least for 3 days at room temperature without agitation and freeze drying could stabilize liposomes for long term storage			Hontoria et al. [170]
PE, CHO, Toc	It is feasible to use liposomes as a delivery system of water soluble antibiotics, oxytetracycline for marine larvae			Touraki et al. [167]
1,2-PA-PC, herring roe PC, CHO	*Artemia* nauplii enrichment with MPL emulsion or MPL-liposomes significantly increased:			McEvoy et al. [165]
	DHA level (% of TL)	DHA:EPA ratio:	Polar lipids content:	
	PT (14%) > SS (6.3%) > L (2%)	PT (1.8) > SS (0.4) > L(0.3)	L (40.1 mg g^{-1}) > PT (32.4 mg g^{-1}) = SS (34.7 mg g^{-1})	
Egg yolk lecithin (60%PC), CHO	Consumption rate of liposomes in gilthead seabream (*Sparus aurata*) and white grouper (*Epinephelus aenaeus*) larvae:			Koven et al. [163]
	Liposomes containing CFE (238.5 ng liposome $larva^{-1}$ n^{-1}) > liposomes containing PHS (54.3 ng liposome $larva^{-1}$ n^{-1})			
	It is feasible to use liposomes as a nutrient supplement in first feeding marine fish larvae			
Crude egg yolk PC (>60%)	Content of methionine in *Artemia* nauplii after different enrichment methods:			Tonheim et al. [168]
Purified egg yolk PC (> 99%), CHO	Purified egg PC liposomes > crude egg PC liposomes > direct enrichment with free methinone > unenriched control			
1,2-PA-PC, Krill PL, soy PC, CHO	Oxidative stability of formulated liposomes:			Monroig et al. [15]
	(100%)Soy PC > (100%)Krill PL			
	(40%)1,2-PA-PC (40%)Krill PL(20%)CHO > (80%)Krill PL(20%)CHO			
	LUV > MLV			
	Addition of CHO improved oxidative stability			
Krill PL (mainly PC, PE)	EFA bioencapsulation depends on methods preparation and structure of vesicles:			Monroig et al. [18]
	LUV detergent > LUV extrusion > MLV extrusion			
Krill PL (mainly PC, PE)	Maximal bioencapsulation is achieved:			Monroig et al. [17]
	Nauplii densities: 300 nauplii ml^{-1}, number of doses of liposomes dispersion: single, product concentration: 0.5 g l^{-1}			
1,2-PA-PC, Krill PL, soy PC, CHO	Types of liposomes, membrane composition (w/w) and findings:			Monroig et al. [19]
	Encapsulation of vitamin A:		Encapsulation of vitamin A:	
	LUV: (98%)Krill PL(2%) vit. A		LUV: (98%)Krill PL(2%) vit. A	
	Increase of retinol content in *Artemia* nauplii		Increase of retinol content in *Artemia* nauplii	
	Encapsulation of methionine:			
	LUV: 80% soy PC20% CHO or 80% 1,2-PA-PC 20% CHO			
	MLV: 80% soy PC20%CHO			
	Efficiency of methionine delivery to *Artemia*: MLV > LUV			
1,2-PA-PC, Krill PL, soy PC, CHO	Oxidative stability of formulated liposomes:			Monroig et al. [16]
	(100%)Soy PC > (80%)1,2-PA-PC (20%)CHO > (80%)soy PC(20%)CHO > (100%)Krill PL (2%) vit A > (100%)Krill PL			
	No size changes of liposomes during the experimental period			

1,2-PA-PC, dipalmitoyl phosphatidylcholine; *PL*, phospholipids; *CHO*, cholesterol; *LUV*, large unilamellar vesicles; *MLV*, multilamellar vesicles; *EFA*, essential fatty acids; *PG*, phosphatidylglycerol; *SS*, Super Selco (Artemia Systems, INV E, Ghent) as control; *PT*, Tuna oil orbital oil emulsified with 12% herring roe polar lipids; *L*, liposomes with the composition, (40%)1,2-PA-PC (40%)PC(20%)CHO; *PS*, phosphatidylserine; *CFE*, cod fish extract; *PHS*, physiological saline; *Toc*, α-tocopherol

variety of enrichment products are available nowadays such as microalgae, microcapsules [158] and oil emulsion products [159].

PL especially MPL are considered to be a better way for providing EPA and DHA for larvae than TAG fish oil due to reasons such as: (1) marine fish larvae commonly ingest and assimilate better natural diets rich in PL than TAG [160–162]. The ratio of DHA:EPA in the PL naturally consumed by larvae is generally higher as compared to the corresponding ratio in TAG fish oil [156], (2) studies also showed that PL facilitate the absorption of lipids in the larvae gut [163] and thus promote growth and survival of larvae [164], and (3) PL have been shown to exert antioxidant properties against oxidation [87, 88].

Mcevoy et al. [14, 165] showed the advantage of using PC from soybean and marine fish eggs in enrichment of *Artemia* nauplii. They found that a mixture of DHA rich fish oil and PC (90:10) resulted in *Artemia* nauplii which were markedly enriched in DHA, and with minimal peroxidation in an aerated mixture during 18 h of enrichment. This is because the added PC functions as a natural emulsifying agent and a natural protectant against oxidation. They also showed that PC from marine egg sources was superior to soy PC in terms of n-3 PUFA content. This is presumably due to the presence of readily assimilable DHA and EPA in a ratio of 2:1 in marine roe lipids as compared to LA in soy PC. Their study corroborated the original work of Kanazawa et al. [166] using soy and bonito PC as feed supplements for larval sea bream and aye.

As mentioned earlier, there are several forms of enrichment products commercially available nowadays for live prey. However, as compared to an emulsion, liposomes provide more advantages. This is due to their ability to encapsulate lipids as well as water soluble components. For example, liposomes have been successfully used to encapsulate vitamin C [19] or water soluble antibiotics [167] in *Artemia* nauplii enrichment. In addition, liposomes can also be used to encapsulate hydrophobic components such as vitamin A [19] and free amino acids such as methionine [19, 168] or glycine [169]. Many studies have also shown that it is possible to encapsulate considerable amounts of n-3 PUFA into liposomes for *Artemia* enrichment [14, 15, 170].

Future Prospects and Conclusion

MPL may offer more advantages to consumer, food, and aquaculture industries as compared to fish oils. Particularly, the use of MPL-based liposomes is expected to provide benefits such as better oxidative stability, higher bioavailability and higher fluidity as compared to other PL-based liposomes. However, the use of MPL-based liposomes is just starting to be explored in both aquaculture and food industries and no current use of MPL-based liposomes for food applications has been reported. The next frontier in liposome application in the food industry will probably focus on the use of MPL for the development of n-3 PUFA enriched functional foods or the use of MPL-based liposomes as nutrient delivery system in foods and feed. Additionally, another area of study that needs further exploration is the use of liposomes for encapsulation of flavor, aroma and natural coloring compound in foods. However, due to the high content of n-3 PUFA in MPL, foods containing MPL are highly susceptible to lipid oxidation, which results in oxidative products that not only cause deterioration of food quality but also increase the risk of certain degenerative diseases as mentioned earlier. Therefore, it is expected that many more studies will be carried out in the future to explore the oxidative stability and sensory properties of MPL or MPL liposomes prior their potential uses in both food and aquaculture industries.

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