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Review

Polyunsaturated fatty acid–cholesterol interactions: Domain formation in membranes

Stephen R. Wassall [a,*], William Stillwell [b]

[a] Department of Physics, Indiana University Purdue University Indianapolis, 402 N. Blackford Street, Indianapolis, IN 46202-3273, USA
[b] Department of Biology, Indiana University Purdue University Indianapolis, 723 W. Michigan Street, Indianapolis, IN 46202-5132, USA

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ABSTRACT

Polyunsaturated fatty acids (PUFA) constitute an influential group of molecules that promote health by an as yet unknown mechanism. They are structurally distinguished from less unsaturated fatty acids by the presence of a repeating =CH–CH_2–CH= unit that produces an extremely flexible chain rapidly reorienting through conformational states. The most highly unsaturated case in point is docosahexaenoic acid (DHA) with 6 double bonds. This review will summarize how the high disorder of DHA affects the properties of the membrane phospholipids into which the PUFA incorporates, focusing upon the profound impact on the interaction with cholesterol. Results obtained with model membranes using an array of biophysical techniques will be presented. They demonstrate DHA and the sterol possesses a mutual aversion that drives the lateral segregation of DHA-containing phospholipids into highly disordered domains away from cholesterol. These domains are compositionally and organizationally the opposite of lipid rafts, the ordered domain enriched in predominantly saturated sphingolipids "glued" together by cholesterol that is believed to serve as the platform for signaling proteins. We hypothesize that DHA-rich domains also form in the plasma membrane and are responsible, in part, for the diverse range of health benefits associated with DHA.

Contents

1. Introduction

While in general the importance of membrane structure cannot be doubted, details of how membranes function at a molecular level remain elusive. In fact, despite the solid foundation laid by decades of membrane research [1–3], understanding the mechanisms of membrane function is one of the major outstanding problems confronting the life sciences. Membranes surround every living cell that has ever existed and were likely an essential component of the first cell. If membranes are so important and ubiquitous, why then do we know so little about them? A typical membrane is overwhelmingly complex, consisting of as many as 1500 different lipid species and a hundred or more different proteins all in rapid flux [4].

* Corresponding author. Tel.: +1 317 274 6908; fax: +1 317 274 2393.
E-mail address: swassall@iupui.edu (S.R. Wassall).

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Even if we could be certain of the exact location of every molecule in a membrane at a given instant, a microsecond later everything would have changed. Due to the large number of chemical species involved and the vast range of size- and time-scales required to study membranes, a wide variety of often-esoteric experimental techniques must be simultaneously employed.

One approach has been to investigate membrane structure from the perspective of a single component [5]. It is the approach that our laboratories have taken and is the focus of this review. We study how polyunsaturated fatty acids (PUFA), particularly docosahexaenoic acid (DHA, 22:6$^{\Delta 4,7,10,13,16,19}$) shown in Fig. 1, affect membrane structure, dynamics and function. DHA is of special interest because with 22 carbons and 6 double bonds it is the most unsaturated and one of the longest fatty acids comprising the membrane hydrophobic core [6]. We believe that this unusual fatty acid, while exhibiting some properties similar to other more common, shorter and less unsaturated membrane fatty acids, will nevertheless alter membrane structure in a unique and measurable fashion. In addition DHA represents the extreme member of a family of fatty acids known as ω3 PUFA that are believed to provide relief for a wide variety of human health problems [7,8]. An abbreviated list of these reputed benefits is found in Table 1. They can be roughly separated into some 6 non-exclusive categories: heart disease, cancer, immune function, neuronal function, aging and "other" hard to categorize problems such as migraine headaches, malaria and sperm fertility [7]. While some links of DHA and health are well established, others are the result of a single report and are at best controversial. Nevertheless it is clear that DHA levels do exert profound effects on significant physiological processes. The elusive question is how one seemingly simple molecule can do so many different things. We postulate that at least a portion of the benefits attributed to DHA can be ascribed to its role in membrane dynamics and structure.

2. Membrane structure

It is now universally accepted that biological membranes are not homogeneous mixtures of lipid and protein but instead are composed of rapidly changing arrangements of widely differing membrane patches called domains [1–3]. Domains are proposed to exhibit different sizes, stabilities, lipid and protein compositions, and functions. Some domains are macroscopic and stable for extended time periods and so are isolatable and well defined. Most of the membrane, however, is likely composed of an enormous number of poorly understood and less stable lipid microdomains [2]. While there are compelling reasons to believe that lipid domains exist, and they have been clearly demonstrated in model lipid monolayer and bilayer membranes, they are difficult to study, and so far, impossible to isolate in pure form from biological membranes [2,3]. Unfortunately, most demonstrations of lipid domains depend on equivocal interpretation of biophysical measurements.

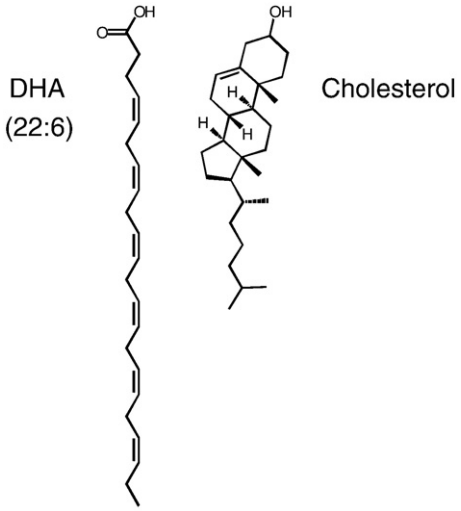

Fig. 1. Molecular structure of docosahexaenoic acid (DHA) and cholesterol.

Table 1
Human conditions affected by DHA levels

ADHD	Depression	Multiple sclerosis
Aggression	Dermatitis	Neurovisual developmental disorders
Alcoholism	Diabetes	Nephropathy
Alzheimer's disease	Dyslexia	Periodontitis
Arthritis	Eczema	Phenylketonuria
Asthma	Fertility	Placental function
Atrial fibrillation	Gingivitis	Psoriasis
Autism	Heart disease	Respiratory diseases
Bipolar disorder	Hypersensitivity	Schizophrenia
Blindness	Inflammatory response	Sperm fertility
Blood clotting	Kidney disease	Suicide
Bone mineral density	Lupus	Ulcerative colitis
Brain development	Malaria visual acuity	Visual acuity
Cancer	Methylmelonic acidaemia	Zellweger's syndrome
Crohn's disease	Migraine headaches	
Cystic fibrosis	Mood and behavior	

Formation of lipid domains is the consequence of unequal affinities between different lipid species or between lipids and membrane proteins. Lipid domain size has been proposed to range between tens of molecules or so (1–10 nm) to large domains on the μm scale [2]. The most studied lipid domains are the result of lateral phase separation between gel and liquid crystalline clusters in protein-free phospholipid monolayers and bilayers. Domains resulting from fluid–fluid phase separations, while much harder to demonstrate, are likely the most significant domain types in biological membranes [9]. By far the best documented of the fluid–fluid domains are phospholipids/cholesterol mixtures that have been shown to segregate into cholesterol-rich (liquid-ordered, l_o) and cholesterol-poor (liquid-disordered, l_d) lipid domains [10] and sphingomyelin (SM)-rich phase-separated membranes [11,12]. If lipid domains do indeed exist in a living membrane, it is reasonable to predict that each distinct domain would attract different proteins and so would support different biochemical functions. In addition, domain boundaries would be locations of high membrane instability and so would support increased permeability, flip-flop, fusion, enzyme activities, etc. [5]. The cell's biochemistry and physiology would therefore directly reflect domain structure and composition, and the domains in turn would be affected by dietary fatty acids.

Recently, there has been considerable interest in one reputed type of lipid domain termed a raft [11,13]. Rafts are sphingolipid and cholesterol-rich l_o domains floating in a sea of l_d phospholipids [11–15]. Their biological interest stems from a close association of rafts with essential cell signaling proteins including lipid-linked proteins such as the src family of kinases in the inner leaflet and GPI-anchored proteins in the outer leaflet of plasma membranes [3,11,16]. An original hallmark of rafts is their insolubility in cold non-ionic detergents, often Triton X-100. The isolated detergent-insoluble fractions (a.k.a. detergent resistant membranes or DRM) are indeed enriched in cholesterol and sphingolipid and significantly contain characteristic membrane signaling proteins [3,11,16]. While DRM mark the beginnings of raft studies, they are fraught with artifacts and are being replaced by gentler, detergent-free methods [17]. Although the small (and apparently ever decreasing) size of domains in biological membranes [2,3] precludes unequivocal imaging, their existence has been inferred by a variety of biophysical techniques including NMR, ESR, fluorescence, X-ray diffraction, and differential scanning calorimetry (DSC) [4]. It is likely, moreover, that numerous proteins may migrate between domains in response to alterations in membrane lipid composition. This concept has been at the heart of our research.

3. DHA in membranes

In mammals DHA is found in extraordinarily high concentrations, occasionally exceeding 50 mol% of the total acyl chains, only in select tissues [6] that include synaptosomes [18], sperm [19] and the retinal rod outer segment [20]. Dipolyunsaturated phospholipids with DHA at both *sn*-1 and -2 positions have even been identified in these tissues [21,22]. The already high DHA levels in these specialized membranes are not further augmented by diet and, once incorporated, DHA is tenaciously retained at the expense of other fatty acids [6]. In sharp contrast to the few tissues containing high levels of DHA are the other tissues where DHA is normally found well below 5 mol% of the total acyl chains. In these tissues DHA can be enriched 2–10 fold through dietary supplementation with foods, notably fish oils, rich in this ω3 PUFA [6,23]. Here DHA would primarily be found in the *sn*-2 chain of phospholipids with the *sn*-1 chain mainly composed of the saturated fatty acids palmitic (16:0) or stearic (18:0) acid [24]. It is DHA's auspicious dietary augmentation in the "low-DHA" membranes that we believe may be responsible in part for the reputed health benefits of DHA.

It has been well documented that DHA is rapidly incorporated into a variety of cells, primarily into phospholipids of the plasma membrane [25] and mitochondria [26,27]. However, DHA is not distributed equally in all phospholipid classes. In most reported studies DHA was shown to be preferentially esterified to PE (phosphatidylethanolamine) with lesser amounts to PC (phosphatidylcholine) and other phospholipid classes [28,29]. DHA-accumulation into PE, however, is not a hard-and-fast rule as Salem et al. [6] have reported that in synaptosomal membranes DHA is often found associated with PS (phosphatidylserine). DHA-containing PE has also been shown to preferentially accumulate in the PE- and PS-rich inner leaflet of several membranes [29–31]. The implication is that DHA-containing phospholipids are heterogeneously distributed in membranes, and that domains rich and poor in this PUFA exist.

This review will address two basic questions about the function of DHA in membranes. First we will briefly discuss whether DHA is indeed different than another very common but much less unsaturated fatty acid, oleic acid (OA, $18{:}1^{\Delta 9}$). We will then address in much greater detail the interaction of DHA with the most common lipid in mammalian plasma membranes, the sterol cholesterol.

4. DHA vs. OA

To better understand the mechanism of DHA-induced membrane phase separations that will be discussed later, we begin by comparing the physical properties of DHA to those of the most common unsaturated fatty acid, OA. A repeating =CH–CH_2–CH= unit is what distinguishes PUFA from less unsaturated fatty acids. This pattern has a profound impact on molecular conformation that was demonstrated by the energy-minimized structures modeled in the pioneering work by Applegate and Glomset [32,33]. The shallow potential energy well for rotation about the single C–C bonds that separate the unsaturated carbon atoms in this pattern produces an extremely flexible, highly disordered structure [34]. DHA chains isomerize so rapidly that they explore their entire conformational space within 50 ns [35]. The torsional states adopted include bent configurations that bring even the lower portions of the PUFA chain up to the membrane surface [36–38]. As a result DHA-containing phospholipids "pack loosely" [5], have a tendency to form non-lamellar phases [39] and can induce negative curvature strain to adjacent membrane lipids and proteins [40]. These properties are not associated with OA-containing phospholipids.

There have been numerous studies reporting the effect of DHA-containing phospholipids on basic physical properties of lipid bilayers and monolayers. A few of them, comparing hetero-acid saturated (16:0 or 18:0)-unsaturated PC with DHA or OA at the *sn*-2 position, are summarized below and listed in Table 2. The reported properties are divided into three categories:

Table 2
A summary comparing the physical properties of DHA- and OA-containing PC

Physical property	DHA[a]	OA[b]	Ratio DHA:OA	Ref.
Physical dimensions				
Area/molecule, monolayer (Å^2)	70[c]	63[d]	1.11	[41]
Area/molecule, bilayer (Å^2)	69.2	61.4	1.13	[42]
Bilayer permeability				
Water (μm/s)	412	155	2.66	[45]
Carboxyfluorescein (% leakage)	35	15	2.33	[46]
Dithionite (s^{-1})	0.017	0.005	3.40	[47]
Erythritol ($d(1/A)dt \times 10^{-2}$ s^{-1})	8.1	3.9	2.08	[46]
Mechanical properties				
Fusion (pmol/10^6 cells)	24.8	2.9	8.55	[46]
Flip-flop (h^{-1})	2.4	0.061	39.34	[47]
Elasticity/compressibility, bilayer (K_a dyne/cm))	121	221	0.55	[42]
Elasticity/compressibility, monolayer (C_s^{-1} mN/m)	108[c]	123[d]	0.88	[41]

[a] 18:0–22:6PC unless otherwise stated.
[b] 18:0–18:1PC unless otherwise stated.
[c] 16:0–22:6PC.
[d] 16:0–18:1PC.

4.1. Physical dimensions

Smaby et al. [41] used pressure/area isotherms on monolayers to demonstrate that 16:0–22:6PC (70 Å^2) occupies an area about 11% larger than 16:0–18:1PC (63 Å^2). Employing a combination of NMR and X-ray diffraction, Koenig et al. [42] measured a similar differential in molecular area in bilayers. They reported an area for 18:0–22:6PC (69.2 Å^2) that is about 13% larger than 18:0–18:1PC (61.4 Å^2). These observations and a subsequent high precision X-ray determination of comparably large surface area per molecule for 18:0–22:6PC (68.2 Å^2) [43] are consistent with the surprising fact that membranes rich in DHA, such as the rod outer segment membrane where DHA levels approach 50% of the total acyl chains, are actually quite thin [44]. The tremendously high disorder of DHA chains, causing them to reorient through a large cross-sectional area, reduces their effective length [36–38].

4.2. Bilayer permeability

One would expect that poorly packed and thin membranes should also be quite permeable. This prediction was confirmed by several different measurements. By ^{17}O NMR Huster et al. [45] saw that bilayers composed of 18:0–22:6PC are 2.7× more permeable to water than 18:0–18:1PC. Higher permeability for 18:0–22:6PC than 18:0–18:1PC was similarly followed by carboxyfluorescein leakage (2.3×) [46], dithionite permeability (3.4×) [47] and erythritol swelling (2.1×) [46].

4.3. Mechanical properties

Substituting DHA for OA has also been shown to have a large effect on the basic membrane properties of fusion, flip-flop and elastic compressibility. In a comparison of fusion rates in sonicated unilamellar vesicles (SUV) composed of either 18:0–22:6PC or 18:0–18:1PC with cultured T27A tumor cells, the DHA-containing vesicles were found to be 8.6× more fusogenic than the OA-containing vesicles [46]. Using dithionite bleaching of NBD-PE to monitor flip-flop in large unilamellar vesicles (LUV), an enhancement of 39× in the rate for flip-flop was seen when 18:0–22:6PC replaced 18:0–18:1PC [47]. Finally, the lateral compressibility modulus for DHA- vs. OA-containing PC was compared by two different methods. Koenig et al. [42] introduced polyethylene glycol (PEG) to apply osmotic gradients that impart lateral pressure to the membrane and determined compressibility on the basis of a combination of order parameter and lamellar repeat spacing derived from ^2H NMR and

X-ray diffraction, respectively. They reported that 18:0–22:6PC was about 1.8× more compressible (lower modulus) than 18:0–18:1PC. Results in qualitative agreement, indicating 16:0–22:6PC is about 1.1× more compressible than 16:0–18:1PC, were obtained from pressure-area isotherms on monolayers by Smaby et al. [41].

From the experiments summarized in Table 2, it is obvious that the multiple double bonds that DHA possesses in addition to OA have a profound effect on membrane structure [48]. The phenomenal flexibility of DHA chains produces a much more dynamic membrane interior. DHA-rich membranes are thin, fluid and loosely "packed", tremendously compressible, very permeable and support high rates of membrane lipid flip-flop [48]. Increased water permeability, for example, makes membranes enriched in DHA "wetter" [45] than OA-rich membranes. The elevated fusion rates reported for DHA-containing model membranes [46] are consistent with the presence of the PUFA in biological membranes known to naturally exhibit high levels of fusion or the reverse process of exfoliation (sperm, rod outer segment and synaptosomes) [6]. Therefore, on the basis of these and other similar experiments we conclude that DHA confers upon membranes dynamic characteristics that the far more abundant OA cannot.

Since all lipids undergo rapid lateral diffusion in the membrane leaflet plane [49], during their excursion through the membrane DHA-containing phospholipids must encounter hundreds of other lipid species. The affinity or aversion that DHA has for these lipids has the potential to drive the formation and to determine the eventual stability of PUFA-rich membrane domains. Cholesterol is the lipid that has been of particular interest to us. DHA–cholesterol interactions and the segregation into domains they promote will be the focus of the remainder of this review.

5. DHA has an aversion for cholesterol

Cholesterol consists of a rigid tetracyclic ring structure with a hydroxyl group at one end and a short hydrocarbon tail at the other (Fig. 1). The sterol is abundant in mammalian plasma membranes, accounting for as much as 50% total lipid [50]. It globally modulates molecular organization and the unequal affinity it possesses for different lipids drives the formation of domains [51]. The interaction of cholesterol with phosphoplipid, especially PC, model membranes has been extensively examined [52]. When incorporated into homoacid disaturated PC membranes, the rigid steroid moiety disrupts the regular packing of chains in the gel (a.k.a. solid ordered, s_o) phase and restricts the reorientation of the fatty acid chains in the lamellar liquid crystalline L_α (a.k.a. l_d) phase [53]. The differential between the phases is smeared out with increasing amounts of sterol until l_o phase, characterized by rapid reorientation but high conformational order, is adopted over a wide range of temperatures at concentrations >25 mol%. Excess sterol is expelled after the content exceeds >50 mol% [54]. Within the membrane, the steroid moiety lines up approximately parallel to the acyl chains with the hydroxyl group situated near the aqueous interface and the short side-chain extended towards the centre of the membrane [55]. There is rapid rotation about the long molecular axis that wobbles through a narrow range of angles slightly tilted relative to the bilayer normal [56]. Heteroacid saturated-monounsaturated PCs exhibit comparable behavior in the presence of cholesterol [57–59].

The relatively few studies published on heteroacid saturated–polyunsaturated PC membranes imply that the addition of cholesterol has the same general effect as seen in homoacid disaturated and heteroacid saturated-monounsaturated PCs [59–61]. Like membranes where the *sn*-2 chain is saturated or monounsaturated, PC membranes with a PUFA chain at the *sn*-2 position become more ordered in the physiologically relevant liquid crystalline state and the sterol reorients rapidly around its "wobbling" long molecular axis. This qualitative resemblance in behavior belies the cholesterol-phobia possessed by PUFA. The high disorder of polyunsaturated chains is responsible. Their rapidly changing conformation pushes away the rigid steroid moiety of a neighboring cholesterol molecule [62]. The predominantly all-trans configuration adopted by a saturated chain, on the contrary, presents a smooth façade that is compatible with intimate contact with the sterol [62]. Recent experimental and computer modeling work support this view. In ^{1}H MAS NMR experiments on 18:0–22:6PC/[25,26,26,26,27,27,27-$^2H_7$]cholesterol (1:1 mol) membranes, closer contact with the 18:0 *sn*-1 chain is revealed by a higher rate of chain-to-cholesterol nuclear Overhauser enhancement cross-relaxation [63]. Molecular dynamics (MD) simulations on 18:0–22:6PC/cholesterol (3:1 mol) corroborate that the sterol favors solvation by saturated over polyunsaturated chains [64]. As there is a series of 9 saturated carbons prior to the double bond in OA that facilitates near approach to the steroid moiety [65], the monounsaturated fatty acid would not be expected to share the poor affinity for cholesterol associated with PUFA. An ability for the sterol to pack well around the monounsaturated, as well as the saturated, chain in 16:0–18:1PC is indeed indicated by recent MD simulations [66].

Measurements of sterol-induced condensation of lipid monolayers give an early example of indirect experimental evidence that cholesterol has an aversion for PUFA. The percentage reduction in average molecular area with respect to the (ideal) weighted average of the molecular areas of the individual components at a given surface pressure serves as an indicator of relative strength of interaction. Whereas cholesterol condenses monolayers made from disaturated and heteroacid saturated-unsaturated PCs containing OA, heteroacid saturated-unsaturated PCs containing DHA do not condense [67,68]. That rapid inter-conversion between conformational states imbued by the recurring =CH–CH_2–CH= motif in PUFA deters close proximity to the steroid moiety is confirmed by a comparison of monolayers prepared from isomeric PCs with 18:0 acid at the *sn*-1 position and polyunsaturated α-linolenic (α18:3) or γ-linolenic (γ18:3) acid at the *sn*-2 position [69]. Appreciable vs. negligible area condensation is exerted by the sterol on 18:0–α18:3PC and 18:0–γ18:3PC, respectively. The determinant is whether the sequence of double bonds occurs below, as for the 9, 12 and 15 positions in the α-isomer, or above, as for the 6, 9 and positions in the γ-isomer, the depth to which the steroid moiety penetrates the monolayer. Partition coefficients recently measured for cholesterol in large unilamellar vesicles (LUV) using a cyclodextrin assay offer a more definitive estimate of strength of interaction [70]. They are smaller in vesicles composed of PC with DHA than less unsaturated chains such as OA, the value for dipolyunsaturated 22:6–22:6PC being even smaller than for 18:0–22:6PC with only a single PUFA chain.

The first unequivocal demonstration of poor affinity for cholesterol was provided by the greatly reduced solubility seen in PUFA-containing membranes. This finding was initially inferred from the small intensity of NMR signals acquired from cholesterol mixed in equimolar amount with dipolyunsaturated 22:6–22:6PC and 1,2-diarachidonylphosphatidylcholine (20:4–20:4PC) membranes [59,63]. Most of the sterol did not contribute to the signal because it was outside the membrane, which contrasts with less unsaturated membranes that can accommodate ≥50 mol% cholesterol [54]. A quantitative estimate of the reduction in solubility was obtained in solid state ^{2}H NMR work, followed-up by X-ray diffraction experiments [59,71–74]. Both methods detect sterol that has been excluded from a membrane to measure the solubility [71]. The NMR-based result is based upon a comparison of the integrated intensity of signals simultaneously observed from membrane-incorporated and -excluded deuterated analogs of cholesterol. In the case of X-ray diffraction, the solubility limit above which cholesterol is excluded from the membrane is revealed by the concentration of added sterol at which diffraction peaks due to solid cholesterol monohydrate begin to appear. A graphical comparison of measurements on DHA-

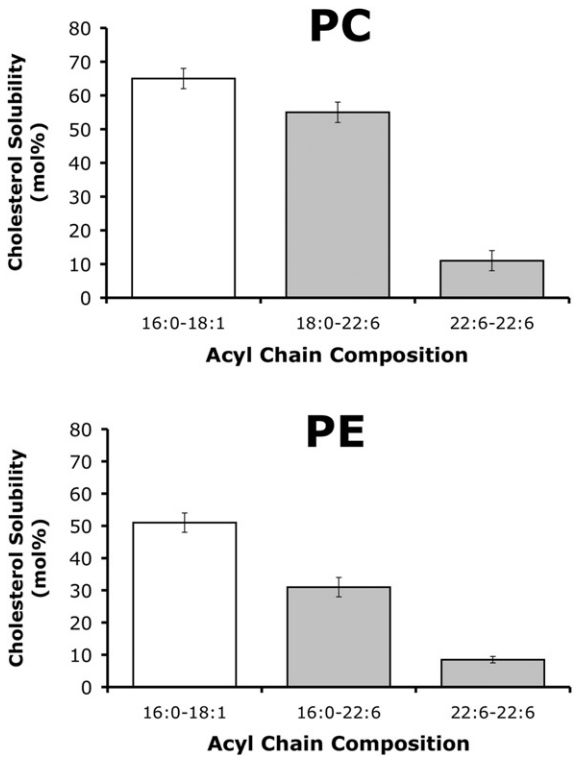

Fig. 2. Solubility of cholesterol in OA- and DHA-containing PC (top panel) and PE (bottom panel) membranes. All samples were in the lamellar liquid crystalline state except 22:6–22:6PE that was in the inverted hexagonal H_{II} phase. Values were taken for 16:0–18:1PC from Huang et al. [54], for 18:0–22:6PC and 22:6–22:6PC from Brzustowicz et al. [72], and for 16:0–18:1PE, 16:0–22:6PE and 22:6–22:6PE from Shaikh et al. [74].

and OA-containing PC (upper panel) and PE (lower panel) membranes is presented in Fig. 2. Reduced values are clearly associated with the PUFA. The reduction is accentuated for PC and PE possessing DHA at both *sn*-1 and -2 positions, revealing that the sterol is pushed out of these membranes where PUFA chains cannot be avoided at a concentration >3× lower than in the mixed chain saturated–polyunsaturated equivalents [72,74]. As opposed to 18:0–22:6PC with a saturated *sn*-1 chain that can accommodate 55±3 mol % cholesterol despite having a DHA chain attached at the *sn*-2 position, there is decreased solubility in heteroacid saturated–polyunsaturated 16:0–22:6PE (31±3 mol%) as well as homoacid dipolyunsaturated 22:6–22:6PE [74]. The additional sensitivity of PE is ascribed to its smaller headgroup and resultant smaller cross-sectional area. This more closely packed arrangement would to tend to force sterol and PUFA together within the membrane, exacerbating their mutual adverse interaction and exclusion of the sterol from the membrane.

Neutron scattering experiments elaborate the unique nature of the aversion cholesterol has for PUFA [58,75]. This experimental approach relies on the difference in sign and magnitude of the scattering amplitude for deuterium and hydrogen. Scattering length density (SLD) calculated from the difference between profiles obtained with labeled and unlabeled analogs of cholesterol incorporated into aligned multilayers identify the location of the labeled position(s) on the deuterated analog as a pair of peaks symmetrically placed about the middle of the bilayer. In the generally accepted arrangement where the long molecular axis of the sterol lines up parallel to the fatty acids chains in each leaflet of the bilayer, the label on [2,2,3,4,4,6-$^2H_6$] cholesterol (head-labeled analog) appears near the aqueous interface approximately ±16 Å from the bilayer center while that on [25,26,26,26,27,27,27-$^2H_7$]cholesterol (tail-labeled analog) is at the center. Remarkably, the results obtained with aligned multilayers of 20:4–20:4PC completely devoid of saturated fatty acid reveal that the center of mass of deuterium in both head- and tail-labeled analogs resides in the middle of the membrane. The PUFA chains push away cholesterol so strongly that, as schematically represented in Fig. 3, the sterol is tipped over to lie flat between leaflets. There the molecule rapidly reorients about its long axis, undergoing whole body motion that is constrained within ±6 Å [75].

A propensity for PUFA to push cholesterol over from its usual orientation, in which the hydroxyl group of the steroid moiety is anchored at the aqueous interface, to the interior of the membrane would facilitate flip-flop of the sterol from one side to the other of a membrane. This notion is supported by recently published coarse grained (CG) simulations on arachidonic acid-containing PC bilayers [76]. In these simulations enhanced rates of flip-flop were observed for cholesterol and the presence of sterol embedded between monolayers was also identified. The implication is that poor affinity for PUFA has the potential to affect the trans-membrane, as well as the lateral, distribution of cholesterol in plasma membranes. PUFA preferentially incorporate into phospholipids, such as PE, that are more abundant in the inner leaflet [31] and would drive the transfer of cholesterol to the outer layer where sphingolipids are primarily found [16]. A substantial redistribution of cholesterol to the outer leaflet, moreover, has been reported following accumulation of PUFA into plasma membranes [77].

6. DHA aversion for cholesterol promotes the formation of domains

The poor affinity that PUFA have for cholesterol drives, we hypothesize, the formation of domains concentrated in PUFA-containing phospholipids but depleted in the sterol [5,10,48,78]. The highly disordered environment within these domains is the absolute antithesis of the ordered one that exists in lipid rafts enriched in predominantly saturated sphingolipids and cholesterol [62,79]. Because dipolyunsaturated phospholipids are most incompatible with cholesterol, they have the greatest tendency to separate into

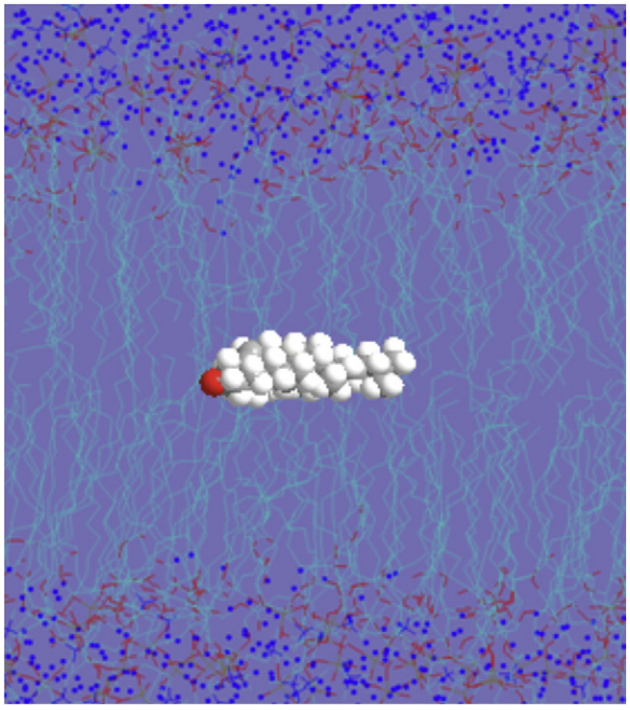

Fig. 3. Schematic representation of the tipped-over orientation for cholesterol in dipolyunsaturated 20:4–20:4PC membranes discovered by neutron scattering [58,75]. The sterol lies flat between leaflets in the middle of the bilayer.

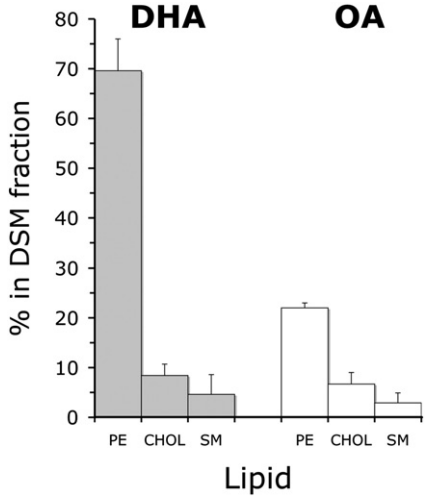

Fig. 4. Percentage of PE, cholesterol (CHOL) and SM in the DSM fraction of 16:0–22:6PE/egg SM/cholesterol (1:1:1 mol) (left) and 16:0–18:1PE/egg SM/cholesterol (1:1:1 mol) (right) membranes treated with 1% Triton X-100 at 4 °C. Each value is relative to the total amount of the specific lipid in detergent-soluble and -insoluble fractions [81].

domains away from the sterol. Fluorescence energy transfer (FRET) measurements on bovine rhodopsin reconstituted into 16:0–16:0PC/22:6–22:6PC/cholesterol bilayer mixtures imply cholesterol induces separation of 16:0–16:0PC into sterol-enriched domains and of 22:6–22:6PC into clusters around the protein [80]. These experiments employed lipids labeled in the headgroup with pyrene as donor and the retinal group of rhodopsin as acceptor. Higher FRET efficiency for the dipolyunsaturated species was detected only in the presence of the sterol. A radius of 35 Å was the size envisaged for the clusters of 22:6–22:6PC molecules with the protein, equivalent to two annular layers of lipid. This radius is compatible with the size of 20:4–20:4PC domains estimated on the basis of ^{2}H NMR data for 18:0–20:4PC/20:4–20:4PC/[3α-$^2H_1$]cholesterol (1:1:2 mol) [71]. The spectrum for the mixed membrane was interpreted in terms of [3α-$^2H_1$]cholesterol partitioning into 18:0–20:4PC and 20:4–20:4PC domains in accordance with its solubility of 49 and 17 mol% in each lipid, respectively. An upper limit of <160 Å was then assigned to the domains assuming that lateral diffusion mediates fast exchange (at a rate greater than the differential in quadrupolar splitting for [3α-$^2H_1$]cholesterol in sterol-poor and -rich domains) between the regions.

Dipolyunsaturated phospholipid domains that are depleted of cholesterol have been proposed to provide the l_d environment necessary for the function of rhodopsin in the disk membrane of the rod outer segment [80]. Apart from neural [18,20,21] and a few other specialized [19,22] membranes where DHA is abundant, however, dipolyunsaturated phospholipids with PUFA *sn*-1 and -2 chains are unlikely. Most plasma membranes contain only modest levels of PUFA that, even when appreciably elevated by dietary consumption, are solely esterified to the *sn*-2 position while a saturated fatty acid is retained at the *sn*-1 position [5]. Poor affinity of cholesterol for PUFA also drives these mixed chain PUFA-containing phospholipids to segregate away from the sterol into domains. Mixtures of 16:0–22:6PE, or 16:0–18:1PE as a control, with the lipid raft molecules SM and cholesterol are the focus of attention in our investigations with heteroacid saturated–polyunsaturated phospholipids. PE, which after PC is the second most common phospholipid in mammalian plasma membranes [4], was chosen for the DHA-containing phospholipid because it is preferred over PC for uptake of the PUFA [25].

DSC of 16:0–22:6PE or 16:0–18:1PE in 1:1 mol mixtures with SM shows that there is separation into PE-rich and SM-rich regions with both DHA- and OA-containing phospholipids [81,82]. Two distinct transitions centered at 8 and 24 °C are seen in the DSC scan for 16:0–22:6PE/egg SM (1:1 mol) [81]. They are attributed to the melting of phases rich in 16:0–22:6PE and SM, respectively. The de-mixing is incomplete since the transition temperatures differ from those for the individual lipids (T_m=2.2 °C for 16:0–22:6PE [83] and T_m=39.0 °C for egg SM [84]). A similar interpretation applies to the DSC scan for 16:0–18:1PE/egg SM. (1:1 mol) [81]. In this case the two transitions overlap into a single broad exotherm, which may be deconvoluted to reveal a transition at T_m=26.1 due to a 16:0–18:1PE-rich phase and another at T_m=30.1 °C due to a SM-rich phase. Addition of cholesterol to either 16:0–22:6PE/SM or 16:0–18:1PE/SM in 1:1:1 mol concentration smears out the transitions beyond detection, indicating that the sterol interacts with PE as well as SM in both mixed systems [81,82]. A substantial differential in interaction of cholesterol with DHA- and OA-containing phospholipids is apparent when a low level of sterol added. The higher-temperature, SM-rich peak in the DSC scan for 16:0–22:6PE/egg SM/cholesterol (1:1:0.005 mol) is obliterated while the lower-temperature, 16:0–22:6PE-rich peak is unaffected [83]. In contrast, the enthalpy of the broad single peak observed for 16:0–18:1PE/egg SM (1:1 mol) is greatly diminished by the same amount of sterol [83].

Greater separation from cholesterol for DHA- than OA-containing phospholipids is observed in 1:1:1 mol mixtures with SM by detergent extraction and solid state ^{2}H NMR. The detergent extraction method consists of incubating an aqueous dispersion of membranes in the presence of a non-ionic detergent and then separating detergent insoluble and soluble fractions by centrifugation [81,85]. Although subject to potential artifacts [86], it is widely used to assess the presence of domains in membranes and insolubility in cold non-ionic detergents has historically served as a definition for lipid rafts [3,11,16]. We compared phase separation between DRM, designated raft, and detergent soluble membranes (DSM), designated non-raft, in 16:0–22:6PE/egg SM/cholesterol (1:1:1 mol) vs. 16:0–18:1PE/egg SM/cholesterol (1:1:1 mol) membranes [81]. The results of experiments conducted at 4 °C, where most detergent solubility studies are performed, are plotted in Fig. 4. Qualitatively similar results were obtained at 40 °C in experiments undertaken to more closely mimic physiological temperature. In agreement with raft molecules being detergent resistant, Fig. 4 confirms that the DSM fraction for both mixtures contains very little SM (<5%) or cholesterol (<10%). That a considerable difference does exist in the amount of 16:0–22:6PE (70%) and 16:0–18:1PE (22%) present in the DSM fraction is clear. The aversion of PUFA for cholesterol is strong enough to cause the majority of the DHA-containing phospholipid to partition into non-raft domains that can be physically separated. The OA-containing phospholipid, which does not possess such poor affinity for the sterol, is not excluded to the same extent from rafts.

The conclusion from DSC and detergent extraction experiments that DHA-containing PE has a greater propensity than OA-containing PE to segregate away from cholesterol in PE/SM mixed membranes is corroborated by solid state ^{2}H NMR work that, furthermore, gives insight into the molecular architecture of domains. In these studies analogs of PE ([$^2H_{31}$]16:0–22:6PE or [$^2H_{31}$]16:0–18:1PE) with a perdeuterated *sn*-1 chain and SM ([$^2H_{31}$]-*N*-palmitoylsphingomyelin, [$^2H_{31}$]16:0SM) with a perdeuterated amide-linked acyl chain are employed to probe the molecular organization of each lipid in PE/SM (1:1 mol) mixtures without and with cholesterol (1:1:1 mol) [81,82]. A comparison of average order parameters $\overline{S}_{CD}$ derived from spectra recorded at 35 °C is presented in Fig. 5. The smaller values measured for [$^2H_{31}$]16:0–22:6PE vs. [$^2H_{31}$]16:0SM and [$^2H_{31}$]16:0–18:1PE vs. [$^2H_{31}$]16:0SM in their respective mixed systems are attributed to the formation of PE-rich, characterized by lower order, and SM-rich, characterized by higher order, domains in the absence and presence of cholesterol in both DHA- and OA-containing membranes. Although order within PE- and SM-rich domains increases when sterol is added to the two systems, the difference in order between them becomes almost a factor of 3 bigger with DHA (0.208 vs. 0.291) than OA (0.266 vs. 0.296). The aversion DHA, but not OA, has for

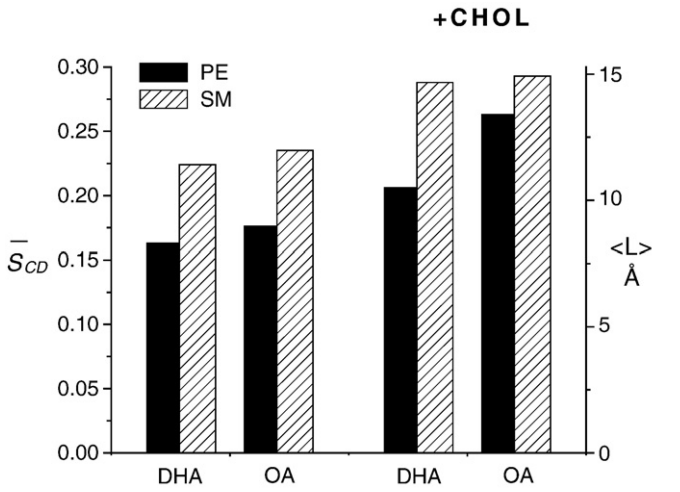

Fig. 5. Average order parameters $\overline{S}_{CD}$ (left hand vertical scale) and average chain length $\langle L \rangle$ (right hand vertical scale) derived from ^2H NMR spectra for [$^2H_{31}$]16:0–22:6PE/egg SM (1:1 mol) and 16:0–22:6PE/[$^2H_{31}$]16:0SM (1:1 mol) and for [$^2H_{31}$]16:0–18:1PE/egg SM (1:1 mol) and 16:0–18:1PE/[$^2H_{31}$]16:0SM (1:1 mol) in the absence and presence of cholesterol (1:1:1 mol) at 35 °C [82].

cholesterol is proposed to be the reason. The sterol is excluded from DHA-containing PE-rich domains and DHA is excluded from SM-rich/sterol-rich domains, thus accentuating the distinction in order between the domains. Physical insight into what the greater differential in order means is revealed by estimates of the average length $\langle L \rangle$ (right hand vertical scale) for [$^2H_{31}$]16:0 chains calculated from the $\overline{S}_{CD}$ values (left hand vertical scale) in Fig. 5. Assuming that $\langle L \rangle$ approximates to the thickness of a monolayer, the divergence between the thickness of the bilayer in SM and PE-rich domains increases from 1.3 to 3.2 Å when DHA replaces OA [82].

Only a single spectral component is discernible for [$^2H_{31}$]16:0–22:6PE, [$^2H_{31}$]16:0–18:1PE or [$^2H_{31}$]16:0SM in the spectra recorded with PE/SM (1:1 mol) and PE/SM/cholesterol (1:1:1 mol) mixtures despite incomplete demixing [81,82]. Therefore, the domains must be small enough for lipid to exchange quickly between them and an upper limit of <200 Å is assigned to the size on the basis of the difference in average quadrupolar splitting in the domains [82]. A comparable radius (<250 Å) was evaluated for 18:0–22:6PC/cholesterol clusters in a similar analysis of ^2H NMR spectra for deuterium labeled phospholipid in 18:0–22:6PC/18:0–22:6PE/18:0–22:6PS/cholesterol (4:4:1:1 mol) [63]. Fast exchange between domains was also inferred from the spectra for deuterium labeled cholesterol in 16:0–18:1PC/brain SM/cholesterol (1:1:1 mol) [87]. The possibility that the presence of PUFA can trigger the formation of large (>μm) from small domains exists. Pulsed field gradient (pfg) NMR measurements on PC/egg SM/cholesterol (3:3:2 mol) membranes aligned between glass plates using PC with a 18:0 acid *sn*-1 chain reveal two lipid lateral diffusion coefficients, ascribed to l_o and l_d phases, when 20:4 or 22:6, but not 18:1 and 18:2, acid is at the *sn*-2 position [49].

The tremendous diversity of disease states alleviated by dietary consumption of PUFA, notably DHA, implies a fundamental mode of action common to most cells. A likely site is the plasma membrane where PUFA incorporate into phospholipids. We hypothesize that lateral sequestration of PUFA-containing phospholipids into domains away from cholesterol is, in part, the molecular origin of the mechanism responsible [5,10,48,62,78]. Fig. 6 illustrates our hypothesis in a cartoon. In the leaflet of a membrane that, for the sake of simplicity, is initially devoid of PUFA there is a lipid raft floating in a sea of bulk lipid (Fig. 6, upper). SM and cholesterol are both enriched in the raft and distributed at low concentration throughout the bulk lipid environment. Replacing bulk lipid by DHA-containing phospholipid creates a DHA-rich (non-raft) domain from which cholesterol is excluded and, together with sphingolipid, driven to further segregate

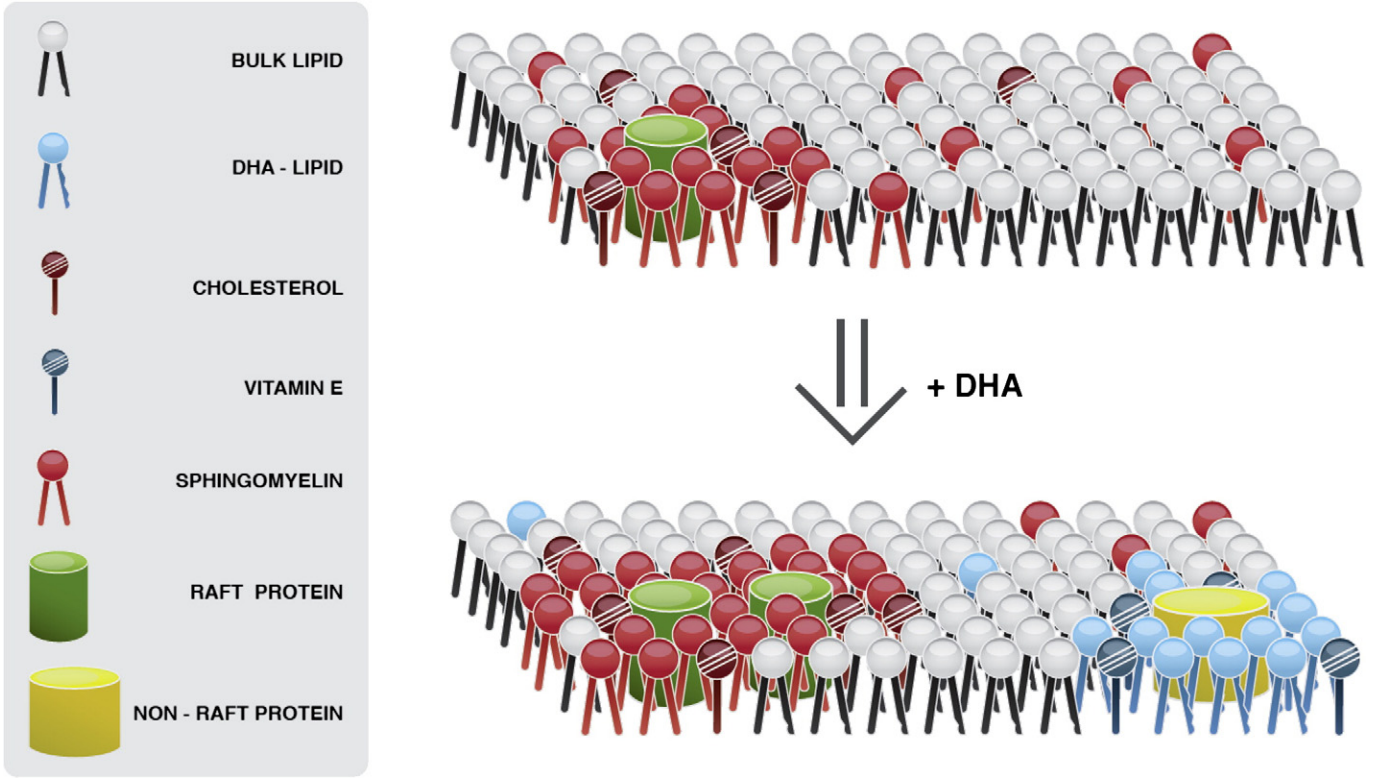

Fig. 6. A cartoon depiction of our proposed model for the molecular mode of action for DHA. Substituting DHA-containing phospholipid for bulk lipid in the plasma membrane creates a DHA-rich non-raft domain from which cholesterol is excluded and further promotes the partitioning of the sterol and SM into a larger lipid raft. The highly disordered DHA-rich domain, the extreme opposite a raft, recruits a protein that requires such an environment to function. This skeletal cartoon is not meant to represent the actual three-dimensional structure of molecules. The purpose is to illustrate, with the aid of color-coding, the segregation of lipids into raft ("red") and non-raft ("blue") domains.

into a larger raft (Fig. 6, lower). A protein requiring a highly disordered environment to function is recruited to the DHA-rich domain, while the enlarged raft can accommodate another resident protein.

A novel paradigm that we now introduce into our model is to postulate a structural role for α-tocopherol, the major component of vitamin E. The presumptive role for α-tocopherol within membranes is to prevent lipid peroxidation that would result in functional impairment [88]. Lipid peroxidation, for instance, contributes to the development of cardiovascular diseases [89,90]. How, when present in only small amount (<1 mol%) [88], the vitamin can efficiently fulfill this role is an unresolved question. As depicted in Fig. 6, we propose that α-tocopherol and DHA-containing phospholipids co-localize in domains. This arrangement would produce a local concentration amplification optimizing protection of the lipid component most susceptible to oxidation. Preferential affinity of α-tocopherol for PUFA, the complete opposite of the situation for cholesterol, is the underpinning mechanism. While strong attraction of the sterol for SM confers stability on lipid rafts, we propose that the vitamin not only prevents oxidation in PUFA-rich non-raft domains but is also the "glue" that holds them together. An appreciation of the strikingly similar yet profoundly different molecular structure of the vitamin and sterol provides insight [91]. Like cholesterol, α-tocopherol consists of a rigid ring structure (chromanol group) that possesses a polar-OH group and a phytyl sidechain. Unlike cholesterol, the smaller chromanol group avoids the adverse interaction with the highly disordered sequence of double bonds in PUFA experienced by the steroid moiety. Experiments are currently underway to test this proposal.

7. Conclusion

PUFA, DHA representing the most extreme example, rapidly interconvert through a wide range of conformations. This high disorder deters close proximity to the rigid steroid moiety of cholesterol, so that PUFA-containing phospholipids tend to push cholesterol away and to laterally segregate into PUFA-rich/sterol-poor domains within membranes. This picture is supported by the work on model membranes reviewed in this article. We hypothesize that the health benefits associated with DHA may be, in part, ascribed to the formation of DHA-rich domains in the plasma membrane. These domains, which are organizationally the antithesis of lipid rafts, recruit and serve as the platform for proteins that require a highly disordered environment to function.

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