RESEARCH ARTICLE

A NEW DETERMINATION OF THE TRANSBILAYER DISTRIBUTION OF PLASMA MEMBRANE CHOLESTEROL

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Synopsis

We propose that the transbilayer distribution of cholesterol in the plasma membrane bilayer is determined by its complexation with the phospholipids in the two leaflets. Because the complexes are homeostatically filled to stoichiometric equivalence, leaflet cholesterol is given by the abundance of its phospholipids multiplied by its sterol stoichiometry. The model predicts that two-thirds of the cholesterol in the human erythrocyte membrane bilayer resides in the outer leaflet. It also predicts the cholesterol content of the bilayer as a whole.

ABSTRACT

The transbilayer distribution of plasma membrane cholesterol remains uncertain despite repeated analysis. We propose a new mechanism driving cholesterol sidedness: sterols form simple stoichiometric associations with phospholipids. Our model postulates that the phospholipids in the plasma membrane bilayer are fully complexed with cholesterol. The cholesterol in each leaflet is then the product of the abundance of its phospholipid and its sterol stoichiometry. Notably, lipid affinities are not relevant. Applying literature values for the composition, abundance and sterol stoichiometry of the phospholipid in each leaflet, the model predicts that two-thirds of the cholesterol in the human erythrocyte membrane bilayer is located in its outer leaflet, an exofacial to endofacial ratio of 2:1. The model also predicts that the overall cholesterol content of the bilayer is ~0.75 mole/mole phospholipid, in agreement with literature values. Furthermore, our analysis suggests that the areas of the two membrane leaflets are about the same. The concordance of prediction with observation validates the model and the values used for the parameters. The sterol in the exofacial leaflet of the plasma membrane of any cell is predicted to exceed that on its contralateral side when its phospholipids have a higher sterol stoichiometry and are fully complexed.

1 | Introduction

The transbilayer distribution of the major lipids in the plasma membrane has been studied extensively, particularly in the human erythrocyte [1-8]. The phospholipids are asymmetrically disposed with almost all the sphingomyelin and phosphatidylcholine located in the outer leaflet and the phosphatidylserine, phosphatidylethanolamine and phosphatidylinositides in the cytoplasmic leaflet [1, 2, 9-11]. However, the transbilayer distribution of the cholesterol remains unresolved. Various studies have suggested that this sterol is located mostly in one leaflet or the other or in a roughly even distribution [3, 6, 12, 13]. Consensus is similarly lacking regarding

the mechanism driving the sidedness of plasma membrane cholesterol. Some have proposed that the outer leaflet has most of the cholesterol because it is enriched in saturated phospholipids with a relatively high sterol affinity [3, 8, 12, 14, 15]. Others have suggested that the sterol is pumped from the inner to the outer leaflet [16, 17]. Another contention is that a relative paucity of exofacial phospholipids is compensated for by extra cholesterol so as to equalize the surface area of the two leaflets and relieve interfacial stress [7, 8]. Alternatively, long chain sphingomyelins in the exofacial leaflet might drive cholesterol to the contralateral side [18]. Theoretical modeling and simulations have implicated the material properties of the bilayer in determining cholesterol sidedness [8, 12-14, 19, 20].

We now propose a different mechanism governing the transbilayer distribution of plasma membrane cholesterol. It is based on the premise that sterols form discrete associations with phospholipids. After McConnell [21], they can be described as reversible noncovalent complexes, possibly multimers of a high order. Their crucial attribute is their stoichiometric equivalence point, manifested by a threshold in the titration of the phospholipid with the sterol [15, 22-24]. While such complexes have not been definitively demonstrated, this assumption is justified in the Discussion.

A new model is proposed for the transverse distribution of plasma membrane sterols, based on experimental values for the asymmetry of their phospholipids. We reason that if the phospholipids in each leaflet are fully complexed with the sterol at their stoichiometric equivalence point, the distribution of the phospholipids will determine that of the sterol. The application of experimental data to the model predicts that two thirds of the cholesterol in the human erythrocyte bilayer is exofacial, a finding consistent with some but not all previous determinations. The stoichiometric complex mechanism differs from some theoretical and computational approaches in doing without the sterol affinity of the phospholipids and

membrane stress. It also offers two results that other treatments lack: it correctly predicts the cholesterol concentration in the membrane as a whole and satisfies the expectation that the areas of the two leaflets are about the same.

2 | Method

The stoichiometric complex model makes the following assumptions. (1) Cholesterol is in diffusional equilibrium within and between the liquid leaflets of the plasma membrane bilayer [24, 25]. (2) Cholesterol associates stoichiometrically with bilayer phospholipids [15, 22, 24, 26]. (3) Plasma membrane cholesterol is maintained homeostatically at the stoichiometric equivalence point of the phospholipids [24, 25, 27]. (4) The molar cholesterol/phospholipid ratio in each leaflet is equal to the stoichiometry of its complexation. (5) Consequently, the abundance of the cholesterol in each leaflet is the product of the abundance of its phospholipid and the stoichiometry of the complexes. (Only when the phospholipids are incompletely filled with cholesterol do their affinities affect their fractional saturation and hence the distribution of the sterol among them.) Glycosphingolipids and low abundance phospholipids are ignored for want of sufficient data.

We define C_t, P_t, C_o, P_o, C_i and P_i as the total (t) moles of cholesterol (C) and phospholipid (P) in the bilayer and in its outer (o) and inner (i) leaflets, respectively. The phospholipids in each compartment are fully complexed with cholesterol at stoichiometries n_o and n_i mole/mole. We express this requirement as C/P = C:P. It follows that the cholesterol in the leaflets are given by,

$$C_o = n_o P_o \qquad (1)$$

$$C_i = n_i P_i \qquad (2)$$

The overall bilayer cholesterol to phospholipid ratio, α is given by:

$$\alpha = C_t/P_t = C_t{:}P_t = (C_o + C_i)/(P_o + P_i) \qquad (3)$$

Then, substituting from Eqs. 1 and 2,

$$\alpha = (n_oP_o + n_iP_i)/(P_o + P_i) \quad (4)$$

Rearranging gives

$$n_o = [\alpha(P_o + P_i) - n_iP_i]/P_o \quad (5)$$

$$n_i = [\alpha(P_o + P_i) - n_oP_o]/P_i \quad (6)$$

$$C_o = n_oP_o = \alpha(P_o + P_i) - n_iP_i \quad (7)$$

$$C_i = n_iP_i = \alpha(P_o + P_i) - n_oP_o \quad (8)$$

Table 1 outlines how the stoichiometric complex model predicts the concentration and transbilayer distribution of sterols in the plasma membrane. The subsequent tables apply the model. Rows 1 and 2 give values assumed for the amount and stoichiometry of the phospholipid in each leaflet. The crux of the model is that because the phospholipids are filled to capacity, the concentration of cholesterol (C/P) in each of the bilayer leaflets is the same as the stoichiometry of its phospholipids (C:P). It follows that the cholesterol content in each leaflet in row 3 is given by the corresponding phospholipid abundance in row 1 multiplied by its stoichiometry in row 2. Row 4 gives the cholesterol concentrations, C/P, by dividing row 3 by row 1. The values for the bilayer as a whole (namely, C_t and C_t/P_t) are calculated from the values for the two leaflets.

Table 1 | The stoichiometric complex model

ROW	PARAMETER	BILAYER	OUTER LEAFLET	INNER LEAFLET
1	Phospholipid, moles P	P_t	P_o	P_i
2	C:P stoichiometry, mole/mole	n_t	n_o	n_i
3	Cholesterol, moles C	n_tP_t	n_oP_o	n_iP_i
4	C/P concentration, mole/mole	C_t/P_t	C_o/P_o	C_i/P_i

3 | Results

We applied the model to the human erythrocyte membrane in Table 2. The abundance of lipids is expressed in moles and the total bilayer phospholipid, P_t, is set at one mole. Concentrations and stoichiometries are in mole/mole. We estimated the distribution of phospholipids between the two leaflets in row 1 by assuming that the outer leaflet is composed entirely of sphingomyelin and phosphatidylcholine [1, 5]. These two species contribute slightly more than half of the total phospholipid [4, 28]. We therefore set the abundance of the phospholipid in the outer leaflet at P_o = 0.50 moles. The inner leaflet would then contain the other phospholipid species plus a few percent each of the phosphatidylcholine and sphingomyelin [1]; they add up to P_i = 0.50 moles.

The outer leaflet stoichiometry (C_o:P_o) in row 2 of Table 2 was assigned a value of 1.0 mole/mole, that determined for its major constituents, phosphatidylcholine and sphingomyelin [26]. The stoichiometry of the complexes in the cytoplasmic leaflet was assigned the value experimentally determined for dioleoylphosphatidylcholine, C_i:P_i = 0.5 mole/mole, taken to represent the unsaturated phospholipids predominant in this leaflet [4, 26]. Since P_o = P_i, the overall stoichiometry of the bilayer is simply the average of C_o:P_o and C_i:P_i ; that is, C_t:P_t = 0.75 (row 2 in Table 2).

The abundance of the cholesterol in each leaflet (row 3) was obtained by multiplying the abundance of the phospholipid in each compartment (row 1) by its corresponding sterol stoichiometry in row 2 (Eqs. 1 and 2). This gives values for the cholesterol in the outer and inner leaflets of C_o = 0.50 mole x 1.0 mole/mole = 0.50 moles and C_i = 0.50 mole x 0.50 mole/mole = 0.25 moles. These are also the values calculated using Eqs. 7 and 8. In addition, they are the differences between the total and the contralateral leaflet cholesterol values, as they must be.

Row 4 gives the cholesterol concentrations (C/P) obtained by dividing the values in row 3 by those in row 1. Concentrations can be converted to mole fractions, C/(C+P).

Table 2 | The stoichiometric complex model applied to human erythrocyte bilayers

ROW	PARAMETER	BILAYER	OUTER LEAFLET	INNER LEAFLET
1	Phospholipid, moles P	1.00	0.50	0.50
2	C:P stoichiometry, mole/mole	0.75	1.00	0.50
3	Cholesterol, moles C	0.75	0.50	0.25
4	C/P concentration, mole/mole	0.75	1.00	0.50

Row 3 in Table 2 delivers the results of interest. The predicted ratio of outer to inner leaflet cholesterol in the human erythrocyte bilayer is $C_o/C_i = 2$. That is, the two leaflets contain 67% and 33% of the cholesterol, respectively. The total bilayer cholesterol concentration is predicted to be $C_t/P_t = 0.75$. This value matches the experimental results for human red cells: $C_t/P_t \approx 0.70$-0.80 [7, 28-31].

We also tested the plausibility of the model by estimating the relative surface areas of the two leaflets of the bilayer using the values in Table 2. If the relative composition of the outer leaflet is taken as 0.50 moles of dipalmitoylphosphatidylcholine plus 0.50 moles of cholesterol, it will have a molecular cross-section of ~39 $Å^2$/molecule at a surface pressure of 30 mN/m, a typical value for plasma membranes [12, 32]. The relative molecular area of the outer leaflet is therefore approximately (0.50 + 0.50) x 39 $Å^2$ = 39 $Å^2$. The endofacial leaflet is less saturated and less condensed than the outer leaflet [4]. If, from Table 2, it is composed of 0.50 moles of dioleoylphosphatidylcholine plus 0.25 moles of cholesterol, it will have a molecular cross-section of ~50 $Å^2$/molecule at a surface pressure of 30 mN/m [12, 33]. The relative molecular area of

the inner leaflet is therefore approximately (0.50 + 0.25) x 50 $Å^2$ = 37.5 $Å^2$. Thus, despite the uncertainties, idealizations and approximations of the estimates, our results meet the expectation that the two bilayer leaflets have closely similar areas.

The tidy outcome in Table 2 may not be accurate or apply to other cells. We therefore tested a few alternative values. In Table 3, we changed the stoichiometry of the outer leaflet in row 2 to 0.90 mole/mole keeping the other parameters the same. The resulting bilayer cholesterol concentration was C_t/P_t = 0.70. This is within the physiological range of $C_t/P_t \approx$ 0.70-0.80 [7, 28-31]. In this scenario, 64% of the cholesterol was exofacial and the cholesterol asymmetry was C_o/C_i = 1.8 instead of 2.0. Thus, the model tolerates modest variation in the stoichiometry of the complexes.

Table 3 | The stoichiometric complex model for bilayers with a different stoichiometry

Row	PARAMETER	BILAYER	OUTER	INNER
1	Phospholipid, moles P	1.00	0.50	0.50
2	C:P stoichiometry, mole/mole	0.70	0.90	0.50
3	Cholesterol, moles C	0.70	0.45	0.25
4	C/P concentration, mole/mole	0.70	0.90	0.50

The abundance of the phospholipids in the two leaflets of the plasma membrane might not always be equal. The cholesterol concentration in the bilayer, C_t/P_t, is then given by the general expression derived from Eq. (3),

$$C_t/P_t = P_oP_i\,(n_i/P_o + n_o/P_i)/(P_o + P_i) \qquad (9)$$

An illustration is provided in Table 4. Here, the values of P_o and P_i in row 1 have been changed to 0.55 and 0.45 moles. Because $P_o \neq P_i$, the value for C_t/P_t in row 2 is not the average of C_o/P_o

and C_i/P_i. As given by Eq. (9), it has the value C_t/P_t = 0.775 mole/mole. This prediction is well within the experimentally observed range of $C_t/P_t \approx$ 0.70-0.80 [7, 28-31]. The ratio of outer to inner leaflet cholesterol in this case is 2.4 with 71% of the cholesterol in the outer leaflet. Thus, the model yields results compatible with the experimentally-determined erythrocyte membrane cholesterol concentration at least over a modest variation in values for the stoichiometry and transbilayer distribution of the phospholipids.

Table 4 | The stoichiometric complex model for bilayers with unequal P_o and P_i

Row	PARAMETER	BILAYER	OUTER	INNER
1	Phospholipid, moles P	1.00	0.55	0.45
2	C:P stoichiometry, mole/mole	0.775	1.00	0.50
3	Cholesterol, moles C	0.775	0.55	0.225
4	C/P concentration, mole/mole	0.775	1.00	0.50

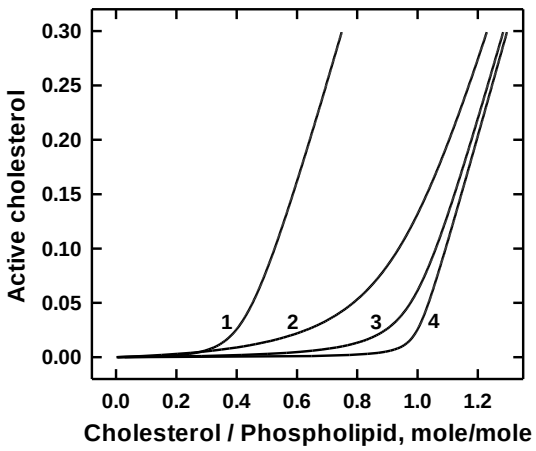
4 | Discussion

We have estimated the transverse asymmetry of cholesterol in human erythrocytes by applying experimental data from the literature to a mechanistic model. It is based on the premise that the bilayer phospholipids are fully complexed with cholesterol. This assumption is supported by the fact that the membrane cholesterol level is at stoichiometric equivalence with the phospholipids; that is, increments above the resting level have a high accessibility to probes characteristic of the uncomplexed form [22, 30, 31]. Furthermore, the concentration of red cell cholesterol, $C_t/P_t \approx$ 0.70-0.80, [7, 28-31], is about that predicted for the sterol capacity of its phospholipids [26].

The model predicts that two thirds of the cholesterol in the human red cell membrane bilayer resides in its outer leaflet (Table 2). The results also fulfill the requirement that the surface areas of the two leaflets be about the same.

We justify the premise that sterols and phospholipids form stoichiometric complexes as follows. The strong association of sterols with phospholipids is manifested by the well known phenomenon of bilayer condensation [21, 34, 35]. This association is driven by the shielding of the sterol molecules from water by an umbrella of phospholipid head groups as well as by favorable van der Waals interactions, hydrogen bonding and some polar contacts [22, 36]. Sterol-phospholipid associations have often been assumed to arise from a favorable energy of interaction or excess Gibbs free energy of solvation that drives sterols to partition into bilayers [37-41]. However, other analyses suggest that sterols and phospholipids do not simply mingle, as is generally the case for the phospholipids themselves; rather, they associate stoichiometrically with stereochemical specificity [21, 22, 33-35, 42-47]. [In this regard, phospholipids have evolved to form stoichiometric complexes not only with sterols but with amphiphiles such as long-chain alcohols [48-51].] Evidence for the existence of these discrete sterol/phospholipid associations is detailed below and illustrated in Figure 1.

(a) Sterol-phospholipid affinity. The relative strength of sterol-phospholipid associations varies with the fatty acyl chains and head groups of the phospholipids as well as with the structure of the sterol [12, 26, 34, 52]. The affinity of phospholipids for cholesterol can exceed that of some binding proteins [53]. How affinity shapes the concentration dependence of the association of sterols and phospholipids is illustrated by curves 3 and 4 in Figure 1. On the other hand, attractive interactions also play a role in other models and are therefore not unique to complexes.



F**IGURE 1 |** Stoichiometric associations of cholesterol and phospholipids in bilayers. Hypothetical isotherms for the association of cholesterol with bilayer phospholipids were generated as previously described [24, 53, 54]. Chemically active (uncomplexed, available) cholesterol is plotted as a function of bilayer sterol concentration. Curve 4 depicts a bilayer with a relatively high cholesterol/phospholipid affinity and a stoichiometry of C:P = 1:1 such as observed for dipalmitoylphosphatidylcholine and sphingomyelin [26]. The sterol remains complexed up to its stoichiometric equivalence point. Curve 3 has the same C:P = 1:1 stoichiometry as curve 4 but a six-fold weaker affinity; it could represent 1-palmitoyl-2-oleoylphosphatidylcholine [26]. (The binding isotherms for proteins that compete with phospholipids for the sterol are similarly shifted to the left of the stoichiometric equivalence point of the lipids [53, 54].) Curve 2 shows competitive displacement of the sterol from complexes by a hypothetical amphiphile such as hexadecanol [50, 51]. The stoichiometry of the association in this example is the same as that in curve 4 (C:P = 1:1) but it has one-thirtieth of its apparent affinity. Curve 1 has a stoichiometry of C:P = 1:2, that reported for dioleoylphosphatidylcholine [26]. Its predicted threshold is therefore C/P = 0.50 but its takeoff is earlier due to the weak affinity assigned.

(b) Sterol-phospholipid stoichiometry. Thresholds such as those pictured in Figure 1 are observed in the titration by sterols of phospholipid monolayers, liposomes and natural membranes [15, 22]. That is, sterols interact poorly with probes and ligands at low bilayer concentrations but become available at a characteristic turning point. (Such probes include cholesterol oxidase [26, 30], methyl-β-cyclodextrin [55, 56], bacterial toxins [31, 54, 57] and a variety of membrane proteins [27, 53, 58-60].) These isotherms presumably reflect two states of the sterol: that complexed with phospholipids and the uncomplexed excess that accumulates in the bilayer beyond the stoichiometric equivalence point. The apparent stoichiometries suggested by such thresholds are typically one or two phospholipids per cholesterol [26]. (This generalization must be qualified by the fact that high affinity ligands can pull the sterol from the phospholipids somewhat below their stoichiometric equivalence point and thereby lower the threshold [53, 54].) Curves 1 and 4 in Figure 1 illustrate isotherms for complexes with stoichiometries of C:P = 1:2 and 1:1 respectively.

(c) Competition and displacement. Many intercalating amphiphiles increase the availability of bilayer cholesterol to probes [49-51, 61, 62]. They apparently associate with the phospholipids themselves, displacing the sterol from complexes mole for mole. A comparison of curves 2 and 4 in Figure 1 illustrates such competitive displacement. Sterol-binding proteins also compete with bilayer phospholipids for cholesterol [53, 54].

(d) Uncomplexed cholesterol has a high chemical potential. The thresholds in isotherms like those in Figure 1 are taken to manifest the transition of the sterol in phospholipid membranes from a low to a high chemical activity near their stoichiometric equivalence point [15, 21, 22, 26, 44, 55]. That thresholds in sterol accessibility in fact represent such a change in its chemical activity has been demonstrated directly [56].

Cholesterol dependence isotherms such as illustrated in Figure 1 cannot be explained by phase changes since they are observed in monolayers and vesicles containing a single phospholipid above its melting temperature [21, 26, 55]. Nor are the thresholds a manifestation of sterol insolubility or crystallization from a super-saturated bilayer solution because the thresholds in chemical activity occur well below the solubility limit (mole fraction ~0.67) and sterol crystals have a low chemical activity [62]. We conclude that isotherms like those in Figure 1 do not represent the nonspecific solvation of sterols in phospholipid bilayers; rather, they manifest their stoichiometric complexation. Whether these entities are discrete and simple or higher-order assemblies is not known but is immaterial to the model.

The model invokes neither membrane stress nor material properties such as intrinsic and spontaneous curvature, inter-leaflet coupling or a difference in leaflet area [8, 12-14, 19, 20]. On the other hand, imposition of any of these factors could work against the bilayer sterol asymmetry created by its complexation. Other models have predicted that most of the sterol is in the outer leaflet based on the high sterol affinity of its exofacial phospholipids [3, 4, 7, 8, 12, 14, 39]. However, there is an important difference between an affinity mechanism and the stoichiometric complex mechanism proposed here, even though the sterol asymmetry in both cases is driven by the preponderance of saturated phospholipids in the outer leaflet. While a differential affinity mechanism can impose an asymmetrical transbilayer distribution, it does not set the abundance of the sterol in the membrane. The stoichiometric complex model predicts both the distribution and the concentration of the sterol in the resting plasma membrane bilayer by including the sterol binding capacity of its phospholipids. For the same reason, the stoichiometric complex model can also predict the cross-sectional areas of the two leaflets.

A recent study has proposed that a fundamentally different mechanism drives excess cholesterol to the exofacial leaflet of the plasma membrane [7]. It envisions that there is a

substantial deficit in the phospholipids in the outer leaflet and that the consequent gap in its surface area is filled by excess cholesterol. An analysis of this model has found it to be insecure [11].

If, as postulated here, nearly all of the plasma membrane sterols and phospholipids are complexed, the complexes would fill both the phase-separated domains (rafts) and the continuum surrounding them. The complexes would thus contribute significantly to the material properties of the membrane: its condensation, molecular order, thickness, permeability, viscosity, compressibility, deformability, various elasticities, intrinsic curvature and lateral phase heterogeneity [7, 8, 34, 63-67]. Other benefits conferred by the saturation of plasma membrane bilayers with sterols include a reduction of their water permeability (especially important for osmotically-vulnerable fresh water eukaryotes such as protozoa) and the imposition of a barrier against the entry of potentially deleterious amphiphiles such as xenobiotics [65, 67, 68].

The uncomplexed fraction of plasma membrane cholesterol, although only a few percent of the total, is chemically active and performs important complementary functions [15, 24, 27, 53, 60, 69-71]. It is the form that diffuses rapidly across the bilayer so as to equalize the chemical activity of the cholesterol in the two leaflets. It is the equilibration of the active plasma membrane cholesterol in erythrocytes with plasma (lipo)proteins that keeps the bilayer phospholipids replete at their stoichiometric equivalence point over months in circulation [7, 25, 28-31]. The cholesterol in the plasma membrane of nucleated cells is similarly buffered by the equilibration of active cholesterol with the plasma (lipo)proteins. In addition, it is maintained metabolically at stoichiometric equivalence with the phospholipids [24, 27, 57]. That is, super-stoichiometric cholesterol circulates to the cytoplasm and provides a homeostatic feedback signal to regulatory elements that keep the plasma membrane phospholipids replete with cholesterol while minimizing any excess. Our model is therefore relevant to the plasma

membranes of nucleated cells. For one thing, their plasma membrane phospholipids have a composition and sidedness similar to those of red cells [2, 4-7, 9, 10, 72, 73]. For another, the cholesterol concentration in the resting plasma membranes of nucleated cells is held close to that in red cells, $C_t/P_t \approx 0.7$. This means that the homeostatic setpoint is near the threshold of their sterol-binding curves; namely, the stoichiometric capacity of their phospholipids [7, 24, 28-31]. (Note that intracellular membranes differ from plasma membranes in that their regulatory elements hold their phospholipids well below capacity [24, 27]. The distribution of the sterol in the cytoplasmic membranes then reflects the affinity of their phospholipids rather than their stoichiometry, and the activity of their regulatory elements will vary with the fractional saturation of their membranes with the sterol.)

Thus, the stoichiometric equivalence point of sterol with phospholipid is a sweet spot. Less sterol undermines the functions mentioned above while the persistence of a super-stoichiometric excess can also be disruptive [29, 65, 74-78].

In conclusion, we anticipate that in any plasma membrane with phospholipids filled to capacity, cholesterol will favor the leaflet with the higher stoichiometry of its complexes; generally, the exofacial side. (This assumes that the abundance of the phospholipids in the two leaflets is similar.) While current evidence encourages this premise, more detailed data for a variety of plasma membranes are needed.

Authors contributions

TLS and YL contributed to every phase of the work from its conception to its publication.

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Ethics statement

Nothing to report.

Conflict of interest

The authors declare no conflict of interest.

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References

1. Murate, M. et al. (2015) Transbilayer distribution of lipids at nano scale. J Cell Sci 128 (8), 1627-38.

2. Murate, M. and Kobayashi, T. (2016) Revisiting transbilayer distribution of lipids in the plasma membrane. Chem Phys Lipids 194, 58-71.

3. Steck, T.L. and Lange, Y. (2018) Transverse distribution of plasma membrane bilayer cholesterol: Picking sides. Traffic 19 (10), 750-760.

4. Lorent, J.H. et al. (2020) Plasma membranes are asymmetric in lipid unsaturation, packing and protein shape. Nat Chem Biol 16 (6), 644-652.

5. Kobayashi, T. (2023) Mapping trasmembrane distribution of sphingomyelin. Emerging Topics in Life Sciences 7 (1), 31-45.

6. Pabst, G. and Keller, S. (2024) Exploring membrane asymmetry and its effects on membrane proteins. Trends Biochem Sci 49 (4), 333-345.

7. Doktorova, M. et al. (2025) Cell membranes sustain phospholipid imbalance via cholesterol asymmetry. Cell 188 (10), 2586-2602 e24.

8. Varma, M. and Deserno, M. (2022) Distribution of cholesterol in asymmetric membranes driven by composition and differential stress. Biophysical Journal 121 (20), 4001-4018.

9. Zachowski, A. (1993) Phospholipids in Animal Eukaryotic Membranes - Transverse Asymmetry and Movement. Biochemical Journal 294, 1-14.

10. Clarke, R.J. et al. (2020) Physiological roles of transverse lipid asymmetry of animal membranes. Biochim Biophys Acta Biomembr 1862 (10), 183382.

11. Lange, Y. and Steck, T.L. (2026) Critique of a radically new model for plasma membrane bilayer organization, in preparation. BioEssays 48:e70114 https://doi.org/10.1002/bies.70114.

12. Aghaaminiha, M. et al. (2021) Quantitative relationship between cholesterol distribution and ordering of lipids in asymmetric lipid bilayers. Soft Matter 17 (10), 2742-2752.

13. Giang, H. and Schick, M. (2016) On the puzzling distribution of cholesterol in the plasma membrane. Chemistry and Physics of Lipids 199, 35-38.

14. Allender, D.W. et al. (2019) Cholesterol-Dependent Bending Energy Is Important in Cholesterol Distribution of the Plasma Membrane. Biophys J 116 (12), 2356-2366.

15. Lange, Y. and Steck, T.L. (2020) Active cholesterol 20 years on. Traffic 21 (11), 662-674.

16. Liu, S.L. et al. (2017) Orthogonal lipid sensors identify transbilayer asymmetry of plasma membrane cholesterol. Nat Chem Biol 13 (3), 268-274.

17. Buwaneka, P. et al. (2021) Evaluation of the available cholesterol concentration in the inner leaflet of the plasma membrane of mammalian cells. Journal of Lipid Research 62, 18.

18. Courtney, K.C. et al. (2018) C24 Sphingolipids Govern the Transbilayer Asymmetry of Cholesterol and Lateral Organization of Model and Live-Cell Plasma Membranes. Cell Rep 24 (4), 1037-1049.

19. Yesylevskyy, S.O. and Demchenko, A.P. (2012) How cholesterol is distributed between monolayers in asymmetric lipid membranes. European Biophysics Journal with Biophysics Letters 41 (12), 1043-1054.

20. Chaisson, E.H. et al. (2023) Building Asymmetric Lipid Bilayers for Molecular Dynamics Simulations: What Methods Exist and How to Choose One? Membranes 13 (7), 629.

21. McConnell, H.M. and Radhakrishnan, A. (2003) Condensed complexes of cholesterol and phospholipids. Biochim Biophys Acta 1610 (2), 159-73.

22. Lange, Y. and Steck, T.L. (2008) Cholesterol homeostasis and the escape tendency (activity) of plasma membrane cholesterol. Prog Lipid Res 47 (5), 319-32.

23. Steck, T.L. and Lange, Y. (2010) Cell cholesterol homeostasis: Mediation by active cholesterol. Trends in Cell Biology 20 (11), 680-687.

24. Steck, T.L. et al. (2021) A basic model for cell cholesterol homeostasis. Traffic 22 (12), 471-481.

25. Estronca, L.M.B.B. et al. (2014) Homeostasis of free cholesterol in the blood: a preliminary evaluation and modeling of its passive transport [S]. Journal of Lipid Research 55 (6), 1033-1043.

26. Lange, Y. et al. (2013) Stability and Stoichiometry of Bilayer Phospholipid–Cholesterol Complexes: Relationship to Cellular Sterol Distribution and Homeostasis. Biochemistry 52 (40), 6950-6959.

27. Lange, Y. and Steck, T.L. (2024) How active cholesterol coordinates cell cholesterol homeostasis: Test of a hypothesis. Progress in Lipid Research 96, 101304.

28. Dodge, J.T. and Phillips, G.B. (1967) Composition of phospholipids and of phospholipid fatty acids and aldehydes in human red cells. J Lipid Res 8 (6), 667-75.

29. Cooper, R.A. et al. (1980) Red cell cholesterol enrichment and spur cell anemia in dogs fed a cholesterol-enriched atherogenic diet. J Lipid Res 21 (8), 1082-9.

30. Lange, Y. et al. (1980) The effect of cholesterol and other intercalated amphipaths on the contour and stability of the isolated red cell membrane. J Biol Chem 255 (19), 9331-7.

31. Chakrabarti, R.S. et al. (2017) Variability of cholesterol accessibility in human red blood cells measured using a bacterial cholesterol-binding toxin. Elife 6.

32. Miyoshi, T. and Kato, S. (2015) Detailed Analysis of the Surface Area and Elasticity in the Saturated 1,2-Diacylphosphatidylcholine/Cholesterol Binary Monolayer System. Langmuir 31 (33), 9086-9096.

33. Dynarowicz-Łątka, P. and Hąc-Wydro, K. (2004) Interactions between phosphatidylcholines and cholesterol in monolayers at the air/water interface. Colloids and Surfaces B: Biointerfaces 37 (1), 21-25.

34. Róg, T. et al. (2009) Ordering effects of cholesterol and its analogues. Biochimica Et Biophysica Acta-Biomembranes 1788 (1), 97-121.

35. Regen, S.L. (2022) Cholesterol’s Condensing Effect: Unpacking a Century-Old Mystery. JACS Au 2 (1), 84-91.

36. Ohvo-Rekila, H. et al. (2002) Cholesterol interactions with phospholipids in membranes. Prog Lipid Res 41 (1), 66-97.

37. Niu, S.L. and Litman, B.J. (2002) Determination of membrane cholesterol partition coefficient using a lipid vesicle-cyclodextrin binary system: effect of phospholipid acyl chain unsaturation and headgroup composition. Biophys J 83 (6), 3408-15.

38. Tsamaloukas, A. et al. (2005) Interactions of cholesterol with lipid membranes and cyclodextrin characterized by calorimetry. Biophys J 89 (2), 1109-19.

39. Nyholm, T.K.M. et al. (2019) The Affinity of Sterols for Different Phospholipid Classes and Its Impact on Lateral Segregation. Biophys J 116 (2), 296-307.

40. Almeida, P.F. (2011) A Simple Thermodynamic Model of the Liquid-Ordered State and the Interactions between Phospholipids and Cholesterol. Biophysical Journal 100 (2), 420-429.

41. Sodt, A.J. et al. (2015) Hexagonal Substructure and Hydrogen Bonding in Liquid-Ordered Phases Containing Palmitoyl Sphingomyelin. Biophysical Journal 109 (5), 948-955.

42. Jedlovszky, P. et al. (2004) Effect of cholesterol on the properties of phospholipid membranes. 3. Local lateral structure. Journal of Physical Chemistry B 108 (1), 465-472.

43. Pandit, S.A. et al. (2004) Complexation of phosphatidylcholine lipids with cholesterol. Biophysical Journal 86 (3), 1345-1356.

44. Radhakrishnan, A. and McConnell, H. (2005) Condensed complexes in vesicles containing cholesterol and phospholipids. Proc Natl Acad Sci U S A 102 (36), 12662-6.

45. Quinn, P.J. (2012) Lipid-lipid interactions in bilayer membranes: Married couples and casual liaisons. Progress in Lipid Research 51 (3), 179-198.

46. Jurak, M. (2013) Thermodynamic Aspects of Cholesterol Effect on Properties of Phospholipid Monolayers: Langmuir and Langmuir-Blodgett Monolayer Study. Journal of Physical Chemistry B 117 (13), 3496-3502.

47. Hąc-Wydro, K. et al. (2007) The study on the interaction between phytosterols and phospholipids in model membranes. Chemistry and Physics of Lipids 150 (1), 22-34.

48. Rowe, E.S. et al. (1998) Thermodynamics of Membrane Partitioning for a Series of n-Alcohols Determined by Titration Calorimetry: Role of Hydrophobic Effects. Biochemistry 37 (8), 2430-2440.

49. Alanko, S.M. et al. (2005) Displacement of sterols from sterol/sphingomyelin domains in fluid bilayer membranes by competing molecules. Biochim Biophys Acta 1715 (2), 111-21.

50. Ratajczak, M.K. et al. (2007) Cholesterol displacement from membrane phospholipids by hexadecanol. Biophys J 93 (6), 2038-47.

51. Lange, Y. et al. (2009) Activation of membrane cholesterol by 63 amphipaths. Biochemistry 48 (36), 8505-15.

52. Rujanavech, C. and Silbert, D.F. (1986) Effect of sterol structure on the partition of sterol between phospholipid vesicles of different composition. J Biol Chem 261 (16), 7215-9.

53. Steck, T.L. et al. (2024) Estimating the Cholesterol Affinity of Integral Membrane Proteins from Experimental Data. Biochemistry 63 (1), 19-26.

54. Lange, Y. et al. (2023) A basic model for the association of ligands with membrane cholesterol: application to cytolysin binding. Journal of Lipid Research 64 (4), 100344.

55. Litz, J.P. et al. (2016) Depletion with Cyclodextrin Reveals Two Populations of Cholesterol in Model Lipid Membranes. Biophys J 110 (3), 635-45.

56. Ayuyan, A.G. and Cohen, F.S. (2018) The Chemical Potential of Plasma Membrane Cholesterol: Implications for Cell Biology. Biophys J 114 (4), 904-918.

57. Das, A. et al. (2013) Use of mutant 125I-perfringolysin O to probe transport and organization of cholesterol in membranes of animal cells. Proc Natl Acad Sci U S A 110 (26), 10580-5.

58. Lange, Y. and Steck, T.L. (2016) Active membrane cholesterol as a physiological effector. Chem Phys Lipids 199, 74-93.

59. Koh, D.H.Z. et al. (2023) Visualization of accessible cholesterol using a GRAM domain-based biosensor. Nat Commun 14 (1), 6773.

60. Ferrari, A. and Tontonoz, P. (2025) Nonvesicular cholesterol transport in physiology. J Clin Invest 135 (6).

61. Regen, S.L. (2023) Taking a Deep Dive into Lipid-Based Mechanisms of General Anesthetics. Biochemistry 62 (7), 1219-1220.

62. Huang, J. (2009) Model membrane thermodynamics and lateral distribution of cholesterol: from experimental data to Monte Carlo simulation. Methods Enzymol 455, 329-64.

63. Hofsäss, C. et al. (2003) Molecular dynamics simulations of phospholipid bilayers with cholesterol. Biophys J 84 (4), 2192-206.

64. Vitkova, V. and Petrov, A.G. (2013) Chapter Five - Lipid Bilayers and Membranes: Material Properties. In Advances in Planar Lipid Bilayers and Liposomes (Iglič, A. and Genova, J. eds), pp. 89-138, Academic Press.

65. Subczynski, W.K. et al. (2017) High Cholesterol/Low Cholesterol: Effects in Biological Membranes: A Review. Cell Biochemistry and Biophysics 75 (3), 369-385.

66. Himbert, S. and Rheinstädter, M.C. (2022) Structural and mechanical properties of the red blood cell's cytoplasmic membrane seen through the lens of biophysics. Frontiers in Physiology Volume 13 - 2022.

67. Khodadadi, E. et al. (2025) Comprehensive Insights into the Cholesterol-Mediated Modulation of Membrane Function Through Molecular Dynamics Simulations. Membranes 15 (6), 27.

68. Wennberg, C.L. et al. (2012) Large influence of cholesterol on solute partitioning into lipid membranes. Journal of the American Chemical Society 134 (11), 5351-5361.

69. Sandhu, J. et al. (2018) Aster Proteins Facilitate Nonvesicular Plasma Membrane to ER Cholesterol Transport in Mammalian Cells. Cell 175 (2), 514-529.e20.

70. Griffiths, W.J. and Wang, Y. (2021) Sterols, Oxysterols, and Accessible Cholesterol: Signalling for Homeostasis, in Immunity and During Development. Front Physiol 12, 723224.

71. Steck, T.L. and Lange, Y. (2023) Is reverse cholesterol transport regulated by active cholesterol? J Lipid Res, 100385.

72. Kiessling, V. et al. (2009) Domain coupling in asymmetric lipid bilayers. Biochim Biophys Acta 1788 (1), 64-71.

73. Doktorova, M. et al. (2020) Structural and functional consequences of reversible lipid asymmetry in living membranes. Nat Chem Biol 16 (12), 1321-1330.

74. Kellner-Weibel, G. et al. (2003) Cytotoxic cellular cholesterol is selectively removed by apoA-I via ABCA1. Atherosclerosis 171 (2), 235-243.

75. van Zwieten, R. et al. (2012) The cholesterol content of the erythrocyte membrane is an important determinant of phosphatidylserine exposure. Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids 1821 (12), 1493-1500.

76. Zhang, X.Y. et al. (2011) Membrane cholesterol modulates the fluid shear stress response of polymorphonuclear leukocytes via its effects on membrane fluidity. American Journal of Physiology-Cell Physiology 301 (2), C451-C460.

77. Allen, D.W. and Manning, N. (1996) Cholesterol-loading of membranes of normal erythrocytes inhibits phospholipid repair and Arachidonoyl-CoA:L-palmitoyl-sn-Glycero-3-Phosphocholine acyl transferase. A model of spur cell anemia. Blood 87 (8), 3489-3493.

78. Feng, B. et al. (2003) The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. Nat Cell Biol 5 (9), 781-92.