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Does complexation of plasma membrane cholesterol with phospholipids determine its transbilayer distribution?

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Research Article

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Abstract

The transbilayer distribution of cholesterol in the plasma membrane is unresolved in that diverse analyses have yielded contradictory results. We now propose a model based on a new mechanism. It assumes that (a) sterols associate stoichiometrically with phospholipids and (b) these complexes are filled to capacity in the plasma membrane. The sterol in each leaflet is then the product of the abundance of the leaflet phospholipids and their sterol stoichiometries. We used literature values for the abundance and sidedness of the phospholipids in each leaflet and for their cholesterol:phospholipid stoichiometries; namely, 1:1 for the exofacial leaflet and 1:2 for the endofacial leaflet. The model predicts that the outer leaflet contains about two thirds of the cholesterol in the human erythrocyte membrane bilayer; i.e., twice that in the inner leaflet. Molecular dynamics simulations have delivered a similar sidedness for membranes in which the exofacial phospholipids have the higher sterol affinity. However, our mechanism holds that sterol affinity is irrelevant if the phospholipids are replete with sterol. Our result matches the observed cholesterol content of the bilayer, ~ 0.75 mole/mole phospholipid (i.e., a mole fraction of ~ 0.43). Furthermore, our results meet the requirement that the areas of the two leaflets be about equal. Generally speaking, in the absence of other factors, the sterol in one leaflet of any membrane bilayer will exceed that of the other when its phospholipids are fully complexed and have higher sterol stoichiometry.

Introduction

The transbilayer distribution of the major lipids in the plasma membrane has been studied extensively, particularly in the human erythrocyte (Doktorova et al., 2025; Kobayashi, 2023; Lorent et al., 2020; Murate et al., 2015; Murate and Kobayashi, 2016; Pabst and Keller, 2024; Steck and Lange, 2018; Varma and Deserno, 2022). It is generally agreed that the polar lipids are asymmetrically disposed with almost all of the sphingomyelin, phosphatidylcholine and glycolipids located in the outer leaflet and phosphatidylserine, phosphatidylethanolamine and phosphatidylinositides in the cytoplasmic leaflet (Clarke et al., 2020; Lange and Steck, 2026; Murate et al., 2015; Murate and Kobayashi, 2016; Steck and Dawson, 1974; Zachowski, 1993).

The transbilayer distribution of the cholesterol remains unresolved. Various studies are in conflict, assigning most of the sterol to one leaflet or the other or suggesting a roughly even distribution (Aghaaminiha et al., 2021; Giang and Schick, 2016; Pabst and Keller, 2024; Steck and Lange, 2018). For example, opposite asymmetries were found when different quenchers were applied to the surface of cells bearing fluorescent sterol surrogates (Doktorova et al., 2025; Mondal et al., 2009; Steck and Lange, 2018; Wood et al., 2011). Consensus is also lacking regarding the mechanism driving the sidedness of plasma membrane cholesterol. It has been proposed that the outer leaflet holds most of the cholesterol because it is rich in saturated phospholipids with a relatively high sterol affinity (Aghaaminiha et al., 2021; Allender et al., 2019; Ingólfsson et al., 2014; Lange and Steck, 2020; Steck and Lange, 2018; Varma and Deserno, 2022). Others have argued that the sterol is pumped from the inner to the outer leaflet (Buwaneka et al., 2021; Liu et al., 2017). Another contention is that extra cholesterol compensates for a

relative paucity of exofacial phospholipids so as to equalize the surface area of the two leaflets and thereby relieve interfacial tension (Doktorova et al., 2025; Varma and Deserno, 2022). It was proposed in one study that the long chain sphingomyelins in the exofacial leaflet drive cholesterol to the contralateral side (Courtney et al., 2018). Another report contended that phosphatidylserine holds the sterol in the endofacial leaflet (Maekawa and Fairn, 2015). In addition, theoretical modeling and simulations have implicated the material properties of the bilayer in determining cholesterol sidedness (Aghaaminiha et al., 2021; Allender et al., 2019; Chaisson et al., 2023; Giang and Schick, 2016; Varma and Deserno, 2022; Yesylevskyy and Demchenko, 2012). Still other factors have been considered (Wood et al., 2011).

We now propose a novel mechanism governing the transbilayer distribution of plasma membrane cholesterol. It assumes that sterols form discrete associations with phospholipids. After McConnell et al., these associations are termed *complexes*. Unlike covalent and coordination complexes, these are presumably rapidly reversible stereochemical associations, such as ligands form with proteins (Anderson and McConnell, 2002; McConnell and Radhakrishnan, 2003; Radhakrishnan and McConnell, 2005; Soloviov et al., 2020). A second assumption is that the phospholipids in the two leaflets of the plasma membrane are filled to capacity with cholesterol. These two features then drive sterol sidedness. That is, in the absence of other factors, the distribution of the phospholipids sets that of the sterol when the phospholipids in each leaflet are fully complexed (i.e., filled to their stoichiometric equivalence point). Evidence supporting this hypothesis is evaluated in the Discussion.

The availability of reliable literature values for the human erythrocyte obviated the need for us to generate experimental data to test the model. These values lead to the prediction that about two thirds of the cholesterol in the bilayer is exofacial. The results are consistent with the measured membrane cholesterol concentration and with equal areas for the two lipid leaflets. An early version of this study has been published as a preprint (Steck and Lange, 2025).

Methods

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 (Estronca et al., 2014; Steck et al., 2021). (2) Cholesterol associates stoichiometrically with the bilayer phospholipids (see Discussion) (Lange and Steck, 2008, 2020; Lange et al., 2013; Steck et al., 2021). (3) Plasma membrane cholesterol is maintained homeostatically at the stoichiometric equivalence point of the phospholipids (Estronca et al., 2014; Lange and Steck, 2024 ; Lange et al., 2014; Steck et al., 2021). The molar cholesterol/phospholipid ratio in each leaflet is then equal to the overall stoichiometry characteristic of its complexes. (4) Consequently, the cholesterol in each leaflet is given by the product of the abundance of its phospholipids and the stoichiometry of their 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.)

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. Thus, the resting cholesterol concentration equals its stoichiometry, denoted as C/P = C:P (mole/mole). It follows that the cholesterol in the two leaflets is given by

$C_o = n_o P_o$ (1)

$C_i = n_i P_i$ (2)

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

$\alpha = C_t/P_t = (C_o + C_i)/(P_o + P_i)$ (3)

Then, substituting from Eqs. 1 and 2,

$\alpha = (n_o P_o + n_i P_i)/(P_o + P_i)$ (4)

Rearranging gives

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

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

$C_o = n_o P_o = \alpha(P_o + P_i) - n_i P_i$ (7)

$C_i = n_i P_i = \alpha(P_o + P_i) - n_o P_o$ (8)

Table 1 outlines how the stoichiometric complex model predicts the concentration and transbilayer distribution of sterols in the plasma membrane. Tables 2–4 then apply the model. Rows 1 and 2 in each table give the values assumed for the amount of the phospholipid in each leaflet and their sterol stoichiometries. 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, obtained 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. The small amounts of glycosphingolipids and minor phospholipids are ignored for want of sufficient data. We similarly disregard sterol-binding proteins; it can be estimated that, even if abundant, their stoichiometric binding of cholesterol would be negligible compared to that of the phospholipids.

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

Results

Table 2 applies the model to the human erythrocyte membrane. The abundance of lipids is expressed in moles, scaled to a total bilayer phospholipid, P_t, of one mole. For row 1, we assumed that the outer leaflet is composed entirely of sphingomyelin and phosphatidylcholine (Kobayashi, 2023; Murate et al., 2015). These two lipids would then contribute slightly more than half of the total phospholipid (Dodge and Phillips, 1967; Lorent et al., 2020). We therefore set the abundance of the phospholipid in the outer leaflet at P_o = 0.50 moles. The inner leaflet will then contain the other phospholipid species plus a few percent each of the phosphatidylcholine and sphingomyelin (Murate et al., 2015); they sum to P_i = 0.50 moles.

The sterol stoichiometries of many of the phospholipids in the human erythrocyte membrane are not known. However, values for ten representative lipids have been determined (Lange et al., 2013). We thereforereasoned as follows: (a) From the preceding paragraph, we assume that the two leaflets have equal amounts of phospholipid. (b) The phospholipids are filled to capacity with cholesterol (see Discussion) (Chakrabarti et al., 2017; Lange et al., 1980; Lange et al., 2013; Lange et al., 2014). (c) The cholesterol content of the plasma membrane is then the weighted average of the stoichiometries of its various phospholipids; i.e., C/P = C:P. (d) The cholesterol content of the human erythrocyte is C_t/P_t ~0.75 moles/mole (Chakrabarti et al., 2017; Cooper et al., 1980; Dodge and Phillips, 1967; Doktorova et al., 2025; Lange et al., 1980). This must also be its stoichiometric ratio; i.e., C_t:P_t ~0.75 moles/mole. (e) Given that the outer leaflet is dominated by phosphatidylcholines and sphingomyelins, it is assigned a stoichiometry of C:P = 1 in row 2 of Table 2 (Lange et al., 2013). (f) We lack a well-defined estimate of the sterol stoichiometry of the cytoplasmic leaflet phospholipids. However, this value is constrained by the stoichiometries of the whole bilayer and its outer leaflet to be C:P ~ 0.5 (row 2 in Table 2). This is reasonable, given that this leaflet is enriched in unsaturated phospholipids (Lorent et al., 2020), at least some of which have this stoichiometry (Lange et al., 2013).

The abundance of the cholesterol in each leaflet (row 3) was estimated by multiplying the abundance of the phospholipid in each compartment in 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 values are also those calculated using Eqs. 7 and 8. 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).

Row 3 in Table 2 delivers the result 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.

Table 2
The stoichiometric complex model applied to the human erythrocyte bilayer

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

Are the areas of the two leaflets equal?

Qualitatively, the outer leaflet has one third more total lipid than the inner leaflet, but it is more condensed by roughly that factor because its phospholipid chains are more saturated. We quantified the relative areas of the leaflets as follows. Two compositions for the outer leaflet were tested: (a) 0.5 moles of dipalmitoylphosphatidylcholine (DPPC) plus 0.5 mole of cholesterol and (b) 0.25 moles of sphingomyelin and 0.25 moles of 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) plus 0.5 moles of cholesterol. The molecular cross-section (area of total lipid) in each case would be ~ 40 $Å^2$/molecule at 30 mN/m, a surface pressure typical of plasma membranes (Aghaaminiha et al., 2021; Edholm and Nagle, 2005; Miyoshi and Kato, 2015; Stottrup et al., 2005). The relative molecular area of the exofacial leaflet is therefore approximately (0.5 moles phospholipid + 0.5 moles cholesterol) x 40 $Å^2$/molecule x NA (Avogadro's number). That is, ~ 40 x NA $Å^2$.

Table 2 specifies the overall stoichiometry of the endofacial leaflet as C:P = 0.5 mole/mole. A phospholipid with this stoichiometry is dioleoylphosphatidylcholine (DOPC) (Lange et al., 2013). In the absence of better alternatives, we took it to represent the phospholipids in the inner leaflet which are known to be rich in unsaturated species (Lorent et al., 2020). This leaflet would then contain 0.25 moles cholesterol + 0.5 moles DOPC. This mixture has an overall molecular cross-section of ~ 52 $Å^2$/molecule at a surface pressure of 30 mN/m (Aghaaminiha et al., 2021; Dynarowicz-Łątka and Hąc-Wydro, 2004; Litz et al., 2016). The relative molecular area of the inner leaflet is then approximately (0.5 moles phospholipid + 0.25 moles cholesterol) x 52 $Å^2$/molecule x NA. That is, ~ 39 x NA $Å^2$. Thus, despite the uncertainties, idealizations and approximations of these estimates, our results meet the expectation that the two bilayer leaflets have closely similar areas.

Tests of the model

The tidy outcome in Table 2 may not be accurate or apply to other cells. We therefore tested alternatives in Tables 3 and 4. We changed the stoichiometry of the outer leaflet in row 2 of Table 3 to 0.90 mole/mole without changing the other parameters. The resulting bilayer cholesterol concentration was $C_t/P_t = 0.70$. This is within the physiological range of C_t/P_t; namely, 0.70–0.80 (mole fraction 0.41–0.44) (Chakrabarti et al., 2017; Cooper et al., 1980; Dodge and Phillips, 1967; Doktorova et al., 2025; Lange et al., 1980). In this scenario, 64% of the cholesterol is exofacial and the cholesterol asymmetry would be $C_o/C_i = 1.8$ 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 an alternative stoichiometry

Row	Parameter	Bilayer	Outer leaflet	Inner leaflet
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. In this case, the cholesterol concentration in the bilayer, C_t/P_t, is given by the general expression derived from Eq. (3),

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

An illustration is provided in Table 4. Here, the values of P_o and P_i in row 1 were 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 derived from 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 ~0.70–0.80 (mole fraction 0.41–0.44) (Chakrabarti et al., 2017; Cooper et al., 1980; Dodge and Phillips, 1967; Doktorova et al., 2025; Lange et al., 1980). The ratio of outer to inner leaflet cholesterol in this case would be 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 within a modest range 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 leaflet	Inner leaflet
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

Discussion

The transverse distribution of cholesterol in the plasma membrane bilayer and the mechanism by which it is established remain controversial despite numerous studies (see Introduction). We approached the issue with a new hypothesis and experimental values from the literature. Our hypothesis is that the plasma membrane bilayer phospholipids are held fully complexed with cholesterol at their stoichiometric equivalence point. That the phospholipids in the red cell bilayer are, in fact, filled to capacity is shown by the dramatic rise in the chemical activity (accessibility) of cholesterol incremented slightly above its resting level (Chakrabarti et al., 2017; Lange et al., 1980; Lange et al., 2013). The plasma membranes of nucleated cells are similarly replete in the sterol (Das et al., 2013; Lange et al., 2014).

The model predicts that two thirds of the cholesterol in the human red cell membrane bilayer resides in its outer leaflet (Table 2). The result cannot be considered definitive, given that we do not know the overall stoichiometry of the diverse phospholipids in the two leaflets. However, assuming stoichiometries of C:P = 1.0 and 0.5 for the two leaflets delivers C_t/P_t = 0.75 mole/mole, a match for that experimentally determined for this membrane, C_t/P_t ~0.70–0.80 mole/mole (Chakrabarti et al., 2017; Cooper et al., 1980; Dodge and Phillips, 1967; Doktorova et al., 2025; Lange et al., 1980). Future work will reveal whether those simple stoichiometries hold true for the many phospholipids in the two leaflets. In any case, the asymmetry predicted here agrees with that delivered by an incisive experiment based on the quenching of a fluorescent sterol surrogate (Doktorova et al., 2025).

But does cholesterol form complexes with phospholipids?

The strength of this premise is crucial to our model and the failure of molecular dynamics simulations to demonstrate sterol-phospholipid complexation represents a challenge (Aghaaminiha et al., 2021; Almeida, 2011; Chaisson et al., 2023; Varma and Deserno, 2022). However, that sterols associate with phospholipids is clearly seen in the condensation of monolayers and bilayers (McConnell and Radhakrishnan, 2003; Regen, 2022; Róg et al., 2009). Multiple factors have been shown to promote these lipid associations: the shielding of the sterol molecules from water under an umbrella of phospholipid head groups; favorable van der Waals interactions; hydrogen bonding; and polar contacts (Lange and

Steck, 2008; Ohvo-Rekila et al., 2002). When complexation is not considered, these forces are often assumed to simply provide a nonspecific favorable energy of interaction or excess Gibbs free energy of solvation that drives the partition of sterols from water into bilayers (Almeida, 2011; Niu and Litman, 2002; Nyholm et al., 2019; Sodt et al., 2015; Tsamaloukas et al., 2005).

There is, however, diverse evidence that sterols and phospholipids do not simply mingle in the bilayer but associate stoichiometrically with stereochemical specificity. The underlying approaches include nuclear magnetic resonance spectroscopy (Darke et al., 1972), the phase behavior of binary mixtures in Langmuir monolayers (Dynarowicz-Łątka and Hąc-Wydro, 2004; Hąc-Wydro et al., 2007; Jurak, 2013; McConnell and Radhakrishnan, 2003), cryoelectron microscopy (Smothers et al., 2026), computer simulations (Jedlovszky et al., 2004; Pandit et al., 2004; Róg et al., 2009), X-ray lamellar diffraction (Hung et al., 2007), chemical cross-linking (Regen, 2022), inelastic X-ray scattering (Soloviov et al., 2020) and theoretical calculations (Radhakrishnan and McConnell, 2005). Other evidence has been reviewed (Lange and Steck, 2008; Litz et al., 2016; Quinn, 2012; Slotte, 1992; Smothers et al., 2026). Impelling evidence for sterol/phospholipid complexation is the threshold in cholesterol-dependence isotherms; this behavior lacks a plausible alternative explanation (see below). The characteristics of these complexes are described below and illustrated in Fig. 1.

1. (a) Sterol-phospholipid affinity. The relative strength of these associations varies with the fatty acyl chains and head groups of the phospholipids as well as with the structure of the sterol (Aghaaminiha et al., 2021; Lange et al., 2013; Róg et al., 2009; Rujanavech and Silbert, 1986). How affinity shapes the concentration dependence of the association of sterols with phospholipids is illustrated by curves 3 and 4 in Fig. 1. That phospholipids associate strongly with cholesterol is also manifested by the fact that they compete for the sterol with cholesterol binding proteins (Steck et al., 2024). However, these associations are not sterol specific: phospholipids also form stoichiometric complexes with amphiphiles such as long-chain alcohols (Alanko et al., 2005; Lange et al., 2009; Ratajczak et al., 2007; Rowe et al., 1998).
2. (b) Sterol-phospholipid stoichiometry. Turning points in isotherms such as those pictured in Fig. 1 are invariably found in the titration by sterols of the phospholipids in monolayers, liposomes and natural membranes. These thresholds signify that the complexed sterol is poorly reactive to probes and ligands while a super-stoichiometric excess is increasingly available (Lange and Steck, 2008, 2020). Probes include cholesterol oxidase (Lange et al., 1980; Lange et al., 2013; Slotte, 1992), methyl-β-cyclodextrin (Ayuyan and Cohen, 2018; Litz et al., 2016), bacterial toxins (Chakrabarti et al., 2017; Das et al., 2013; Lange et al., 2023) and a variety of membrane proteins (Ferrari and Tontonoz, 2025; Koh et al., 2023; Lange and Steck, 2016; Lange and Steck, 2024; Steck et al., 2024). The stoichiometries suggested by such thresholds

are typically one or two phospholipids per cholesterol (Lange et al., 2013; Litz et al., 2016; McConnell and Radhakrishnan, 2003; Slotte, 1992). (Note that high affinity ligands such as proteins can pull the sterol from the phospholipids; the thresholds of their binding curves therefore fall below the stoichiometric

equivalence point of the sterol (Lange et al., 2023; Steck et al., 2024).) Curves 1 and 4 in Fig. 1 illustrate isotherms for complexes with stoichiometries of C:P = 1:2 and 1:1 respectively.

1. (c) Competition and displacement. Many intercalating amphiphiles increase the availability of bilayer cholesterol to probes and ligands (Alanko et al., 2005; Huang, 2009; Lange et al., 2009; Ratajczak et al., 2007; Regen, 2023). They apparently associate with the phospholipids, displacing the sterol from the complexes mole for mole. A comparison of curves 2 and 4 in Fig. 1 illustrates such competitive displacement. The binding of cholesterol by sterol-specific binding proteins must similarly compete with the bilayer phospholipids, as seen in their sigmoidal sterol-dependence isotherms (Steck et al., 2024). Such competition would not occur if the sterol were not complexed with the phospholipid.
2. (d) Uncomplexed cholesterol has a high chemical potential. The thresholds in isotherms like those in Fig. 1 are taken to manifest the transition of the sterol in phospholipid membranes from a low to a high chemical activity as they approach their stoichiometric equivalence point (Lange and Steck, 2008, 2020; Lange et al., 2013; Litz et al., 2016; McConnell and Radhakrishnan, 2003; Radhakrishnan and McConnell, 2005). That this behavior in fact reflects a change in the chemical activity of cholesterol has been demonstrated directly (Ayuyan and Cohen, 2018). Thresholds in chemical activity are characteristic of complexes but not of simple solutions.

Is there another mechanism for thresholds?

Could the thresholds in the concentration dependence of cholesterol isotherms reflect some other mechanism? For instance, do they reflect phase changes in the bilayer? This is unlikely, given that thresholds are observed in monolayers and vesicles containing a single phospholipid above its melting temperature (Lange et al., 2013; Litz et al., 2016; McConnell and Radhakrishnan, 2003). Nor could such thresholds be a manifestation of sterol insolubility or crystallization from a super-saturated bilayer solution because thresholds in chemical activity occur well below the solubility limit for bilayer cholesterol which is typically C/P = 2.0 (i.e., mole fraction ~ 0.67) (Huang, 2009; Litz et al., 2016). Furthermore, sterol crystals have a low chemical activity while super-stoichiometric membrane cholesterol has a high chemical activity (Ayuyan and Cohen, 2018).

Consideration of other mechanisms driving sterol sidedness

Our model challenges previous conclusions regarding bilayer cholesterol sidedness (see Introduction). Various theoretical treatments have explored the influence on cholesterol sidedness of membrane stress and material properties such as intrinsic and spontaneous curvature, inter-leaflet coupling and differential leaflet area (Aghaaminiha et al., 2021; Allender et al., 2019; Chaisson et al., 2023; Giang and Schick, 2016; Varma and Deserno, 2022; Yesylevskyy and Demchenko, 2012). Our model is parsimonious and does not invoke any of these factors. Future studies might show that their imposition alters the sterol sidedness created by its complexation. On the other hand, these forces do not appear to have much effect on human erythrocyte membranes (Himbert and Rheinstädter, 2022). Other theoretical

studies have assumed that the higher affinity of the exofacial phospholipids underlies the preponderance of the sterol in that leaflet (Aghaaminiha et al., 2021; Allender et al., 2019; Doktorova et al., 2025; Ingólfsson et al., 2014; Lorent et al., 2020; Nyholm et al., 2019; Steck and Lange, 2018; Varma and Deserno, 2022). There is an important difference, however, between that affinity mechanism and the stoichiometric complex mechanism proposed here, even though the sterol asymmetry in both cases is driven by the abundance of saturated phospholipids in the outer leaflet. While a differential affinity mechanism can impose an asymmetrical transbilayer distribution, it does not set the concentration of the sterol in the membrane as does the saturated complex model. Since, as discussed below, plasma membrane cholesterol rests at stoichiometric equivalence with the phospholipids, the complexation mechanism is favored over the affinity mechanism.

A recent publication proposed a novel mechanism that drives cholesterol to the exofacial leaflet of the plasma membrane (Doktorova et al., 2025). It postulates a substantial deficit in the phospholipids in the outer leaflet with the consequent gap in its surface area filled by excess cholesterol. This model has been critiqued (Feigenson, 2025; Lange and Steck, 2026; Skotland and Sandvig).

Other implications of cholesterol-phospholipid complexation

If, as postulated here, the sterols and phospholipids in plasma membrane bilayers are normally close to stoichiometric equivalence, their complexes will fill both phase-separated domains (rafts) and the continuum surrounding them. Complexes would therefore 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 (Doktorova et al., 2025; Himbert and Rheinstädter, 2022; Hofsäss et al., 2003; Khodadadi et al., 2025; Róg et al., 2009; Subczynski et al., 2017; Varma and Deserno, 2022; Vitkova and Petrov, 2013). Particular benefits conferred by the saturation of plasma membrane bilayers with sterols are a low 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 (Khodadadi et al., 2025; Subczynski et al., 2017; Wennberg et al., 2012).

The uncomplexed fraction of plasma membrane cholesterol, although only a few percent of the total, is chemically active and performs important complementary physiological functions (Ferrari and Tontonoz, 2025; Griffiths and Wang, 2021; Lange and Steck, 2020; Lange and Steck, 2024; Sandhu et al., 2018; Steck et al., 2024; Steck and Lange, 2023; Steck et al., 2021). It is the form that rapidly diffuses across the bilayer so as to equalize the chemical activity of the cholesterol in the two leaflets. In the case of the erythrocyte, equilibration of active plasma membrane cholesterol with plasma (lipo)proteins keeps the bilayer phospholipids replete at their stoichiometric equivalence point over months in circulation (Chakrabarti et al., 2017; Cooper et al., 1980; Dodge and Phillips, 1967; Doktorova et al., 2025; Estronca et al., 2014; Lange et al., 1980). The cholesterol in the plasma membrane of nucleated cells is similarly buffered by the equilibration of their active cholesterol with plasma (lipo)proteins. In addition, elaborate metabolic mechanisms maintain the plasma membrane cholesterol at stoichiometric equivalence with

its phospholipids (Das et al., 2013; Lange and Steck, 2024; Steck et al., 2021). That is, super-stoichiometric plasma membrane cholesterol circulates to the cell interior and serves as a feedback signal that regulates the homeostatic elements keeping the plasma membrane phospholipids replete with cholesterol while minimizing any excess.

These considerations suggest that the stoichiometric equivalence point of plasma membrane sterol and phospholipid is a sweet spot. Less sterol undermines the functions mentioned above while the persistence of a super-stoichiometric excess can be disruptive (Allen and Manning, 1996; Cooper et al., 1980; Feng et al., 2003; Kellner-Weibel et al., 2003; Subczynski et al., 2017; van Zwieten et al., 2012; Zhang et al., 2011).

Plasma membrane cholesterol sidedness in other cells

While the profile of plasma membrane phospholipids varies among nucleated cells (Lorizate et al., 2013; Skotland and Sandvig, 2022; Symons et al., 2021), their sidedness is well-regulated (Norris et al., 2024) and resembles that of human red cells (Clarke et al., 2020; Doktorova et al., 2020; Kiessling et al., 2009; Kobayashi, 2023; Lorent et al., 2020; Murate et al., 2015; Murate and Kobayashi, 2016; Pabst and Keller, 2024; Zachowski, 1993). Furthermore, the cholesterol concentration in the plasma membranes of nucleated cells (Calderón and DeVries, 1997; Steck et al., 2021; Symons et al., 2021; van Meer et al., 2008) is similar to that in red cells, C_t/P_t ~0.7–0.8 (Chakrabarti et al., 2017; Cooper et al., 1980; Dodge and Phillips, 1967; Doktorova et al., 2025; Lange et al., 1980). These findings support the hypothesis that the setpoint for cholesterol homeostasis is generally close to the threshold of the plasma membrane sterol-binding curve set by the stoichiometric capacity of its phospholipids (Lange and Steck, 2024; Steck et al., 2021). Intracellular membranes differ from plasma membranes in that their cholesterol levels are held well below the capacity of their phospholipids (Lange and Steck, 2024; Steck et al., 2021). The distribution of the sterol in these membranes therefore reflects the affinity of their phospholipids rather than their stoichiometry. This allows the activity of their homeostatic elements to vary with the fractional saturation of their bilayers with the sterol.

Conclusion

We have estimated the distribution of cholesterol across the human erythrocyte membrane bilayer using a novel model and secure experimental values from the literature. It appears that, in the absence of other influences, the cholesterol in a membrane bilayer will favor the side with the higher stoichiometry if the phospholipids in the two leaflets are fully complexed and of similar abundance. This will generally be the exofacial side. Now needed to test the validity and generality of this hypothesis are the phospholipid compositions, sidedness and sterol stoichiometries of a variety of other plasma membranes.

Declarations

Competing interests

The authors declare no competing or financial interests.

Competing interests

The authors declare no competing or financial interests.

Author Contribution

Theodore Steck and Yvonne Lange contributed equally to the conception of the study and the preparation of the manuscript.

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Figures

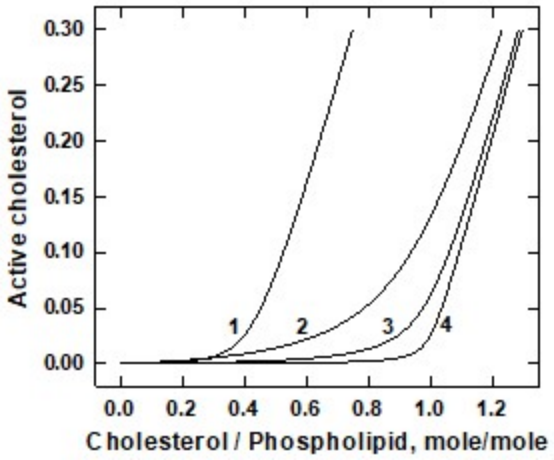


Figure 1

Stoichiometric associations of cholesterol and phospholipids in bilayers. Hypothetical isotherms were generated as previously described (Lange et al., 2023; Steck et al., 2024). Chemically active (uncomplexed, accessible, available) cholesterol is plotted as a function of bilayer sterol concentration. The isotherms are taken to reflect two states of the sterol: that associated with phospholipids and the uncomplexed excess that accumulates in the bilayer at sterol concentrations exceeding the stoichiometric equivalence point. Curve 4 depicts a bilayer with a relatively high phospholipid affinity for cholesterol and a stoichiometry of C:P = 1:1 such as observed for dipalmitoylphosphatidylcholine (DPPC) and sphingomyelin (Lange et al., 2013). The phospholipid in curve 3 has the same 1:1 stoichiometry as curve 4 but a six-fold weaker affinity; hence, an earlier takeoff. It could represent 1-palmitoyl-2-oleoylphosphatidylcholine, POPC (Lange et al., 2013). Curve 2 illustrates competitive displacement of the sterol from phosphatidylcholine complexes by a hypothetical amphiphile such as hexadecanol (Lange et al., 2009; Ratajczak et al., 2007). The stoichiometry of association in this example is the same as that in curves 3 and 4 (namely, C:P = 1:1) but the apparent affinity is much lower. Curve 1 has a stoichiometry of C:P = 1:2, that reported for dioleoylphosphatidylcholine (DOPC) (Lange et al., 2013). Its predicted threshold is therefore C/P = 0.50, but ittakes off early due to the weak affinity of the complex.