Registered and antiregistered phase separation of mixed amphiphilic bilayers

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We derive a mean-field free energy for phase separation in coupled bilayer leaflets. Our model accounts for amphiphile-level structure, in particular hydrophobic mismatch, which promotes antiregistration (AR), in competition with the ‘direct’ trans-midplane coupling usually studied, promoting registration (R). For phospholipid parameters equilibrium demixing is typically R, but hydrophobic mismatch favours metastable AR phases, which can cause a bilayer in the ‘spinodal region’ to require nucleation in order to equilibrate. This provides a framework for understanding existing observations, elucidating a subtle, often overlooked competition of couplings, and a key role for phase transition kinetics in determining domain registration/antiregistration.

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Introduction: Phase separation in amphiphilic bilayers is of great interest due to cellular roles of lipid ‘rafts’ [1,2], and as a means of designing function into artificial membranes. A full understanding of their rich phase behaviour requires consideration of the separate, yet coupled, leaflets of the bilayer [3,4]. Such inter-leaflet coupling is especially important in, e.g., mechanisms of protein localisation via domain formation [2].

Experiment and simulation yield disparate results. Observations of registered domains [10] (Fig. 1a) imply a mismatch free energy per area favouring registration (R), which we call ‘direct’ coupling [11]. However, R domains typically differ in hydrophobic thickness, ∼ 1 nm for liquid-ordered vs. liquid-disordered (LO-LD) phospholipid domains, leading to energetically costly hydrophobic mismatch. Thus an ‘indirect’ coupling promotes antiregistration (AR) to relieve thickness mismatch (Fig. 1b), in competition with the direct coupling. Antiregistration was inferred experimentally at the single-amphiphile level [12], while AR domains have appeared in LO-LD [13] and liquid-gel [14] simulations, and AFM has shown R domains decaying into AR [15].

Despite its wide practical importance, this complex behaviour lacks a full theoretical picture. Existing theories [3,7] treat the bilayer as two phenomenologically coupled phase-separating leaflets, with an order parameter to describe the demixing transition. The phenomenological parameters in these models are not directly related to molecular features of bilayers. Hydrophobic mismatch is often not explicitly included in coarse-grained modelling [8,9,10,15], so that the competition of direct and indirect inter-leaflet couplings described above cannot be captured [17].

We approach the problem using a mean-field free energy derived from a lattice model of coupled leaflets, in which simplified molecular interactions and bilayer structuring, including hydrophobic mismatch, appear explicitly. We show how competing interactions (favouring Fig. 1a, 1b) lead to rich free energy landscapes, thereby unifying observations of registration and antiregistration [10,13,14]. We point out the importance of kinetics, showing how a bilayer can be influenced by metastable antiregistered phases. As a consequence, a bilayer in the conventional ‘spinodal’ region can, paradoxically, require nucleation in order to equilibrate.

Mean-field free energy: To obtain the mean-field free energy, we first consider a lattice model (Fig. 1c) in which a patch of the bilayer is an $L^2 = N^2$ square lattice of sites, where each site contains a ‘top’ (t) and ‘bottom’ (b) leaflet amphiphile. The lattice spacing is the lateral distance between amphiphiles, $a \sim 0.8$ nm for phospholipids [18]. Each amphiphile has a hydrophobic length $\ell^t(b)$. We define the bilayer thickness $d_i = \ell^t_i + \ell^b_i$, and the difference $\Delta_i = \ell^t_i - \ell^b_i$. Extension of the tails also entails greater ordering, which we implicitly map onto the length variables $\ell^t(b)_i$ [19]. We consider species $S$ and

\[ \ell^t(b)_i \]

FIG. 1. (a) Registration (R), satisfying the direct coupling but with thickness mismatch penalised by the indirect coupling. (b) Antiregistration (AR), without thickness variation but a mismatch penalty from the direct coupling. (c) Mesoscopic lattice model for coupled bilayer leaflets.
U to suggest saturated and unsaturated lipids, where S prefers a longer, more ordered tail structure. The species can also represent phases of a ternary (S+U+cholesterol) bilayer on a two phase tie-line [16, 20, 21]. Each lattice site may be ‘pairwise’ registered (SS or SU), or antiregistered (SU or US). The species’ ideal lengths are \( \ell_{SU}, \ell_{US} \). We define \( \Delta_0 \equiv \ell_{SU} - \ell_{US}, \) \( d_0 \equiv \ell_{SU} + \ell_{US} \). We choose \( \ell_{SU} > \ell_{US} \), but this choice is arbitrary.

From the lattice model, incorporating the direct and indirect couplings along with amphiphile stretching and an Ising-like (length-independent) interaction, we derive [18] a mean-field (MF) free energy per site \( \phi \) as a function only of coarse-grained, MF variables for the local average composition \( \phi^{(t)} \equiv N_{\phi}^{(t)} / N \) and thickness of each leaflet. These impose constraints

\[
\sum_\alpha N_\alpha d_\alpha = N \bar{d} , \tag{1a}
\]

\[
\sum_\alpha N_\alpha \Delta_\alpha = N \bar{\Delta} , \tag{1b}
\]

\[
N_{SU} - N_{US} = (\phi^t - \phi^b)N , \tag{1c}
\]

\[
N_{SS} + N_{SU} = \phi^t N , \tag{1d}
\]

\[
N_{UU} + N_{US} = (1 - \phi^t)N , \tag{1e}
\]

where \( N_\alpha \) are the occupancies of the four possible site types \( \alpha \in \{SS, SU, US, UU\} \) with thickness variables \( d_\alpha, \Delta_\alpha \). We find [18] that the desired \( f(\phi^t, \phi^b, \bar{d}, \bar{\Delta}) \) is given by minimising

\[
f' N = \sum_\alpha (N_\alpha H_\alpha + k_B T N_\alpha \ln N_\alpha) - 2VN(\phi^t - \phi^b)^2 - 2VN(\phi^t + \phi^b - 1)^2 , \tag{2}
\]

subject to Eqs. [15,16] where \( H_\alpha \) contain the thickness-dependent MF interactions for each site type

\[
H_\alpha = \frac{1}{2} J(d_\alpha - \bar{d})^2 + \frac{1}{2} B(\Delta_\alpha)^2 + \frac{1}{2} \kappa ((\ell_0^t - \ell_0^b)^2 + (\ell_0^t - \ell_0^b)^2) , \tag{3}
\]

\( \ell_0^t = \ell_{A0} \) and \( \ell_0^b = \ell_{B0} \) for \( \alpha = AB \). The mixing penalty \( V \equiv V_{SU} - \frac{1}{2}(V_{SS} + V_{UU}) \) captures interactions independent of amphiphile length (e.g. headgroup interactions). The hydrophobic penalty \( J \) acts on the total bilayer thickness, ‘indirectly’ coupling the top and bottom amphiphiles of a given site via the surrounding thickness, and favours antiregistration to minimise thickness variation (Fig. [1b]). The ‘direct’ coupling \( B \) promotes registration, similar to the conventional mismatch free energy density \( \gamma \) [3, 4, 22, 23] (see [18]). \( \kappa \) penalises length stretching – we use a value corresponding to an area stretching modulus \( k_A \approx 60 k_B T \bar{d}^{-2} \) [18].

\( f \) can further be minimised over the MF total thickness \( \bar{d} \) and thickness difference \( \bar{\Delta} \) to yield their annealed values \( \bar{d}^{\text{ann.}} = \Delta_0(\phi^t + \phi^b - 1) + d_0 \) and \( \bar{\Delta}^{\text{ann.}} = \kappa \Delta_0(\phi^t - \phi^b) / (2B + \kappa) \). Phase behaviour is governed by

\[
f^{\text{ann}}(\phi^t, \phi^b) \equiv f(\phi^t, \phi^b, \bar{d}^{\text{ann.}}, \bar{\Delta}^{\text{ann.}}) . \tag{4}
\]

Because the free energy is explicitly symmetric under species interchange, tie-line endpoints ‘1’ and ‘2’ in individual leaflets satisfy \( \phi_1^{(b)} = 1 - \phi_2^{(b)} \) (this symmetry does not imply a loss of generality in the qualitative findings). Local bilayer demixing can be \( \phi_1^{(t)} \) or \( \phi_1^{(b)} \), which respectively entail a local preponderance of pairwise R or AR amphiphile pairs.

An example free energy landscape is shown in Fig. 2. For this state point (marked in Fig. 4c), both R and AR demixed minima exist, with R being the equilibrium (lowest free energy) phases. The presence of metastable minima implies nontrivial kinetics for registration and antiregistration (similarly, metastable gas-liquid coexistence can cause complex kinetics in the freezing transition of colloids [24]). Consider, for example, the kinetics of an initial ‘mixed state’ \( \phi^t = 0.5 \), as would exist after quenching a bilayer with an equimolar mixture in each leaflet. In Fig. 2 the mixed state is unstable (i.e. concave down) to both R and AR modes. Although the equilibrium phases are R, metastable AR phases will be accessed first if the AR mode is fastest.

Fig. 3 shows R/AR slices through \( f^{\text{ann}}(\phi^t, \phi^b) \) for some different state points (marked in Fig. 4b). For the lowest direct coupling \( B \), the equilibrium phase is AR demixed. Upon increasing \( B \) (penalising AR), R demixing becomes equilibrium (but with the mixed state metastable against it), and then AR demixing is lost. For the largest \( B \) the mixed state is unstable to R demixing. Fig. 3 also shows that the leaflet compositions of the AR phases do not coincide with the R phases, as they are affected differently by the inter-leaflet couplings. Increasing \( B \) brings the AR demixed minima progressively inward towards \( \phi^t = 0.5 \) until they disappear, but moves the R demixed minima outward, towards \( \phi^t = 0, 1 \).

**Instability criteria:** Instability requires negative curvature of \( f^{\text{ann}} \). For the R/AR modes, we define \( f^{\text{ann}}(\phi^t) \equiv f^{\text{ann}}(\phi^t, \phi^b) \) where \( m = R, AR \). Hence, instability of a given mode \( m \) at the equimolar
by the R (AR) demixing mode. For example, increasing state, for creating excess pairwise R (AR) sites required the loss of configurational entropy, relative to the mixed state, for most amphiphiles aligned with the opposite species. pairwise AR in the mixed state (as measured in [12]), increases the entropy-like part enough to render the mixed state metastable against R demixing (as for the middle two panels of Fig. [3] [23]). J promotes demixing of pairwise R sites once they are created, but also increases the cost of creating R sites in the first place, creating a free energy barrier for R demixing. In contrast, the Ising-like interaction V trivially increases the instability.

**Growth rates:** When, as in Fig. [2] the mixed state is unstable to both R and AR modes, initial demixing will be determined by a competition of the modes. This can be studied by comparing their growth rates in a linear stability analysis of a Ginzburg-Landau (G-L) free energy

$$F_{G-L} = \int d^2 r \left( \frac{f}{a^2} + f_{\text{grad}} \right),$$

arising from the nearest-neighbour interactions that depend on the total thickness and leaflet compositions. \( (J \equiv J/4) \) is the ‘bare’ hydrophobic mismatch coupling in the underlying lattice model [15]. We obtain wavenumber dependent growth rates \( \omega_m(q) \) whose maxima over \( q \) yield \( \omega_{\text{max}}(q) \). The difference \( \Delta \omega = \omega^{\text{R}}_{\text{max}} - \omega^{\text{AR}}_{\text{max}} \) determines which mode dominates the initial demixing.

**Stability diagrams:** Combining the preceding analyses, Fig. [4] shows the dependence of equilibrium phases, R/AR instabilities, and their relative growth rates on direct and indirect couplings, for varying choices of the Ising-like interaction \( V \) and difference in ideal lengths \( \sigma \).

### Weak mismatch \((\sigma = 1 a)\)

The MF Ising model (our model with \( B = J = 0 \)) requires \( V > V_0 \approx 0.5 k_B T \) to demix. For \( V = 0.3 k_B T \) and weak thickness mismatch, no demixing takes place (Fig. [4a]), while \( V = 0.6 k_B T \) (Fig. [4b]) induces demixing as in the MF Ising model. The equilibrium state is R demixed, but both modes are unstable and for strong enough hydrophobic mismatch, the AR mode can be faster (red). Hence, the state point marked in Fig. [4] will initially undergo spinodal demixing in the AR mode, accessing the metastable AR minima (Fig. [2]). The bilayer subsequently requires nucleation to reach the equilibrium R minima, despite having begun in the ‘spinodal’ region.

### Strong mismatch \((\sigma = 2 a)\)

Increasing \( \sigma \) strengthens both the indirect and direct couplings (physically, this could arise from increasing both the degree of length mismatch and difference in saturation of the species’ tails). In contrast to the weak mismatch case, for \( V = 0.3 k_B T \) (Fig. [4b]) the inter-leaflet couplings can induce demixing although (since \( V < V_0 \)) neither leaflet would demix ‘on its own’ [3] (i.e. without coupling). A large hydrophobic penalty \( J \) promotes pairwise AR which, due to the doubled effective Ising interaction between \( SU \) and \( US \) pairs \( (2V > V_0) \), leads to AR demixing. There is even a small region in which AR demixing is the *equilibrium* state. Increasing the direct coupling \( B \) favours R demixing, enhanced by hydrophobic thickness mismatch between \( SS \) and \( UU \) sites – but large \( J \) renders the mixed
Fig. 4. (a)–(d) Stability diagrams in indirect and direct couplings $J, B$, at varying values of the Ising interaction strength $V$ and the difference in ideal thicknesses. Thick lines and bold labels indicate the equilibrium phase $R$, $AR$ or mixed. Thinner lines denote instability of an equimolar mixture to $R/AR$ modes, the labels appearing on the side of the lines to which they refer. **Secondary axis:** approximate values of the inter-leaflet mismatch free energy per area $\gamma$. **Colours:** from linear stability analysis of the equimolar mixture [18], $\Delta \omega > 0$ (R mode faster, blue), $\Delta \omega < 0$ (AR faster, red). In (c) the colour scale range is reduced by a factor 5 from that indicated on (d). **Green dots:** in (b) correspond to Fig. 3, in (c) corresponds to Fig. 2. (e) and (f) show illustrative $q$-dependent growth rates.

**State metastable, not unstable, against equilibrium R demixing** (within the $R$ region but outside the ‘$R$ instability’ line). For $V = 0.6 k_B T$ (Fig. 4d), demixing always takes place since $V > V_0$.

**Discussion:** We have modelled the coupled leaflets of a bilayer in which hydrophobic mismatch can cause an indirect coupling $J$, promoting antiregistration (AR). This competes with a direct coupling $B$, arising from tail structure mismatch, which promotes trans-midplane registration ($R$) of like species. These competing interactions lead to rich free energy landscapes that imply complex kinetics for $R/AR$ demixing. As simulated in [13, 14], increased hydrophobic mismatch favours AR demixing, typically a metastable, not equilibrium, state.

The predictions depend on the interplay of $J$ with $B$, the latter approximately mapping to the inter-leaflet mismatch free energy per area $\gamma$ [18]. For typical phospholipids ($J \sim 2 a^{-2} k_B T$ [18]) with a significant lipid length mismatch $\Delta_0 \sim 0.8 \text{ nm} (\sim 1 a)$, Fig. 4: may apply. Taking $\gamma \approx 0.15 k_B T a^{-2} \sim 0.1 a^{-2} k_B T$ [3, 23] (so $B \approx 0.23 a^{-2} k_B T$) on that figure implies comparable R/AR growth rates. However, [4] argues for $\gamma \sim 0.01 a^{-2} k_B T$ ($B \approx 0.02 a^{-2} k_B T$). Then, the mixed bilayer is likely to first access a metastable AR demixed state, paradoxically requiring nucleation to reach R equilibrium, despite having begun in the spinodal region [28]. Both estimates of $\gamma$ suggest $B < J$, implying the mixed state is mostly pairwise AR (Eq. 7), in agreement with [12].

Hydrophobic mismatch has both intra- and inter-leaflet effects, encouraging demixing but also indirectly coupling the leaflets. This dual role is absent in previous phenomenological theories [3, 7], which *a priori* consider only purely intra- and purely inter-leaflet couplings.

Macroscopic domains should typically be registered at equilibrium, since the hydrophobic mismatch energy scales as domain edge while the direct coupling scales as area [3]. Registration observations in LO-LD [10] may indicate that nucleation barriers to R demixing are typically surmountable. In contrast, AFM experiments [15] have shown small R gel domains converting to an AR state. In light of our results, this could indicate decay of subcritical R nuclei into a metastable AR state. It is likely that metastable AR free energy minima are generically implicated in observations of full or partial...
antiregistration $[13][15]$, and in the apparent dependence of domain registration on thermal history $[15]$. Standard fluorescence microscopy or AFM might not distinguish side-by-side complementary AR domains (SU alongside US), which would be of identical total thickness and similar fluorescence, thus appearing homogeneous and potentially disguising any initial AR demixing step. The rich free energy landscapes of coupled leaflets imply equally rich kinetics for registration and antiregistration, whose study is particularly important given the nonequilibrium nature of the cell membrane. The free energy can also be used to find phase equilibria under given constraints on overall leaflet compositions, permission of flip-flop, etc. We have focused on approximate phospholipid parameters, but the phenomenology also applies to polymeric bilayers $[29][30]$, whose properties and hence predicted phase behaviour may be quite different.

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[25] While laterally mixed, the bilayer can contain more or fewer pairwise R vs. AR sites. At $\phi^R = \phi^D = 0.5$ in particular, anything from full pairwise R to AR is possible.
[26] In principle the mixed state may become metastable against AR demixing if $B \gg J$, but for phospholipids the literature suggests $J \gtrsim B$ or $J \gg B [13]$. Further, the ‘complementary matching’ (pairwise AR) measured in $[12]$ requires, within our model, $J > B$ (via Eq. [7]).
Supplemental Material: Registered and antiregistered phase separation of mixed amphiphilic bilayers

All equations and references in the Supplemental Material have their numbers prefixed with ‘S’. Hence, ‘Eq. 2’ (for instance) refers to Eq. 2 of the main text, while ‘Eq. S2’ refers to the second equation in the Supplemental Material, whose label is ‘S2’. This applies also to figure references which, since no figures exist in the Supplemental Material, always refer to the main text.

### I. MEAN-FIELD SOLUTION

The underlying lattice model is a $L^2 = N$ square lattice of sites with ‘top’ (t) and ‘bottom’ (b) leaflet amphiphiles. The exact lattice Hamiltonian is

$$H = \sum_{<i,j>} (V_{\phi_i^t \phi_j^t} + V_{\phi_i^b \phi_j^b}) + \sum_{<i,j>} \frac{1}{2} J(d_i - d_j)^2 + \sum_i \frac{1}{2} B(\Delta_i)^2 + \sum_i \frac{1}{2} \kappa ((\ell_i^t - \ell_0^t)^2 + (\ell_i^b - \ell_0^b)^2) ,$$

(S1)

where $\ell_0^{(t)} = \ell_0$ for an $S$ amphiphile at the top (bottom) of site $i$, or $\ell_{U0}$ for $U$. For each site, $d_i = \ell_i^t + \ell_i^b$, and $\Delta_i = \ell_i^t - \ell_i^b$. The Ising interactions $V_{..}$ occur among $S$ and $U$ amphiphiles within each leaflet. We define $\phi_i^{(t,b)} = 1, 0$ if the top (bottom) of site $i$ contains an $S, U$ amphiphile. The exact partition function is

$$Z = \sum_{\{\phi_i^{(t,b)}\}} \int \mathcal{D}\Delta \mathcal{D}d \exp(-\beta H) ,$$

(S2)

where the sum is constrained by the average leaflet compositions $\bar{\phi}_i^{(t,b)} = \Lambda_i^{t(b)}/N$, and we have defined $\mathcal{D}\Delta \equiv \prod_i d\Delta_i$ and $\mathcal{D}d \equiv \prod_i d\ell_i$. The free energy is related to the partition function by

$$fN = -k_B T \ln Z ,$$

(S3)

and our aim is to find a mean-field approximation to the free energy per site $f$ that depends only on coarse-grained variables for the composition and thickness of each leaflet of the bilayer.

#### A. Mean-field (on-site) approximation

We use a mean-field approximation, ignoring correlations between neighbouring sites. This requires approximating the neighbour interaction terms of $H$ (those involving $V$ and $J$) with on-site terms. For the $J$ term, we define the mean-field bilayer thickness $d \equiv \sum d_i/N$ and write

$$\sum_{<i,j>} (d_i - d_j)^2 = \sum_{<i,j>} ([d_i - \bar{d}] - [d_j - \bar{d}])^2$$

$$= \sum_{<i,j>} ([d_i - \bar{d}]^2 + [d_j - \bar{d}]^2 - 2[d_i - \bar{d}][d_j - \bar{d}])$$

$$\approx \sum_i 4(d_i - \bar{d})^2 .$$

(S4)

The mean-field approximation consists in assuming the cross term $[d_i - \bar{d}][d_j - \bar{d}]$ to sum to zero, i.e. that $d_i$ and $d_j$ are uncorrelated.

For the Ising-like term in (say) the top leaflet, the interaction matrix $V_{\alpha\beta}$ permits a mapping to the Ising model. Define the exchange parameter $J^{\text{Ising}}$ (unrelated to the $J$ in our model) and the ‘spin’ variable $s_i^t \equiv 2\hat{\phi}_i^t - 1$, which takes the value 1 or $-1$, and consider the Ising model in which the interaction energy between spins $i$ and $j$ is $E_{ij} = -J^{\text{Ising}} s_i^t s_j^t$. In the mean-field approximation, the total energy of this Ising model is $E \approx -2J^{\text{Ising}} \bar{s}^t <\bar{s}^t>^2 N$ where $\bar{s}^t$ is the mean value of the spin. This can be written as

$$E = \sum_{<i,j>} -J^{\text{Ising}} s_i^t s_j^t \approx \sum_i -2J^{\text{Ising}} \bar{s}_i^t \bar{s}^t .$$

(S5)
The excess interaction energy for unlike versus like neighbours in the Ising model is \( E_{ij} \mid s_i^s = -s_i^t \mid E_{ij} \mid s_i^s = s_i^t = 2J_{\text{Ising}} \). For \( V_{\alpha \beta} \), this difference is given by \( V \equiv V_{10} - \frac{1}{2}(V_{00} + V_{11}) \). Hence equivalence with the Ising model is established by setting \( V = 2J_{\text{Ising}} \). Therefore, in the mean-field approximation of our lattice Hamiltonian, we can write

\[
\sum_{\langle i,j \rangle} V_{\phi_i^t \phi_j^t} \approx \sum_i -V s_i^t \hat{s}^t = \sum_i -V s_i^t (2\hat{s}^t - 1),
\]

(S6)

and similarly for the bottom leaflet. The mean-field (i.e. on-site) approximation to the Hamiltonian is thus given by

\[
H \approx H_{\text{MF}} = \sum_i H_i,
\]

(S7)

where

\[
H_i = -V s_i^t (2\hat{s}^t - 1) - V s_i^b (2\hat{s}^b - 1) + \frac{1}{2} J (d_i - \bar{d})^2 + \frac{1}{2} B (\Delta_i)^2 + \frac{1}{2} \kappa ( (\ell_i^t - \ell_0^t)^2 + (\ell_i^b - \ell_0^b)^2 ) ,
\]

(S8)

and \( J \equiv 4\bar{J} \).

### B. Site types

We now note that, given a mixture of \( S \) and \( U \) species in each leaflet, there are four possible site types, \( \alpha \in \{SS, SU, US, UU\} \) where an \( AB \) site contains species \( A \) on the top and \( B \) on the bottom. \( SS \) and \( UU \) sites are pairwise registered, while \( SU \) and \( US \) sites are pairwise antiregistered. All sites of type \( \alpha \) share the same values of the species-dependent constants \( s_i^t, s_i^b, \ell_0^t \) and \( \ell_0^b \) in their \( H_i \). We can therefore express the total mean-field Hamiltonian \( H_{\text{MF}} \) as a sum over the noninteracting site-level Hamiltonians

\[
H_{\text{MF}} = \sum_i H_i = \sum_{\alpha} \sum_{j_{\alpha}} N_{\alpha} H_{j_{\alpha}},
\]

(S9)

where \( j_{\alpha} \) labels the \( j \)th out of \( N_{\alpha} \) sites of type \( \alpha \), and

\[
H_{j_{\alpha}} = \pm V (2\bar{\phi}^t - 1) \pm V (2\bar{\phi}^b - 1) + \frac{1}{2} J (d_{j_{\alpha}} - \bar{d})^2 + \frac{1}{2} B (\Delta_{j_{\alpha}})^2 + \frac{1}{2} \kappa ( (\ell_{j_{\alpha}}^t - \ell_0^t)^2 + (\ell_{j_{\alpha}}^b - \ell_0^b)^2 ) .
\]

(S10)

Here, \( \ell_{0}^t = \ell_{A0} \) and \( \ell_{0}^b = \ell_{B0} \) for \( \alpha = AB \), and the \( \pm \) are --- for \( \alpha = SS, ++ (UU), -- (SU) \) and \( += (US) \).

The sum over the top and bottom species occupancy of sites can be rewritten as a sum over the occupancies of the set of site types, i.e.

\[
\sum_{\{\bar{\phi}_i^t, \bar{\phi}_i^b\}} = \sum_{\{N_{\alpha}\}} \frac{1}{\prod_{\alpha} N_{\alpha}!} ,
\]

(S11)

where the factorials avoid overcounting indistinguishable configurations and the sum is constrained by

\[
N_{SU} - N_{US} = (\bar{\phi}^t - \bar{\phi}^b)N, \quad (S12a)
\]

\[
N_{SS} + N_{SU} = \bar{\phi}^t N, \quad (S12b)
\]

\[
N_{UU} + N_{US} = (1 - \bar{\phi}^t)N. \quad (S12c)
\]

Defining \( D\Delta_{\alpha} = \prod_{j_{\alpha}} d\Delta_{j_{\alpha}} \) and \( Dd_{\alpha} = \prod_{j_{\alpha}} dd_{j_{\alpha}} \), the mean-field partition function \( Z_{\text{MF}} \) is

\[
Z_{\text{MF}} = \sum_{\{N_{\alpha}\}} \frac{1}{\prod_{\alpha} N_{\alpha}!} \prod_{\alpha} \int D\Delta_{\alpha} Dd_{\alpha} \exp (-\beta \sum_{j_{\alpha}} H_{j_{\alpha}}) .
\]

(S13)
Since all sites are now independent of one another, the integral may be rewritten in terms of the partition function for a single site of type $\alpha$. Additionally, the constraints Eqs. S12a–S12c allow the Ising interaction $V$ to be factored out. We thus have

$$Z_{MF} = \sum_{\{N_\alpha\}} \exp \left( -\beta N V^*(\bar{\phi}^t, \bar{\phi}^b) \right) \prod_\alpha Z_{\alpha}^{N_\alpha},$$

where we have defined

$$V^*(\bar{\phi}^t, \bar{\phi}^b) \equiv -2V(\bar{\phi}^t - \bar{\phi}^b)^2 - 2V(\bar{\phi}^t + \bar{\phi}^b - 1)^2.$$  \hfill (S15)

The single-site thickness partition function is given by

$$Z_\alpha = \int d\Delta_\alpha dd_\alpha \exp (-\beta H_\alpha),$$

in which

$$H_\alpha = \frac{1}{2}J(d_\alpha - \bar{d})^2 + \frac{1}{2}B(\Delta_\alpha)^2 + \frac{1}{2}\kappa (d_\alpha^2 - \bar{d}_\alpha^2)^2 + (\bar{d}_\alpha - \bar{d}_0^2)^2,$$

now contains only the thickness-dependent interactions.

C. Self-consistency, free energy

For self-consistency of the average bilayer thickness $\bar{d}$ and the average difference $\Delta$, we require the integrations over $d_\alpha, \Delta_\alpha$ to be performed such that

$$\sum_\alpha N_\alpha d_\alpha = N\bar{d},$$ \hfill (S18a)
$$\sum_\alpha N_\alpha \Delta_\alpha = N\Delta.$$ \hfill (S18b)

Since these integrals are Gaussian, and the constraints Eqs. S18a–S18b are linear, the integrations can be performed exactly to yield

$$\prod_\alpha Z_{\alpha}^{N_\alpha} = \exp (-\beta \sum_\alpha N_\alpha H_\alpha \{d^*_\alpha, \Delta^*_\alpha\}),$$ \hfill (S19)

where $\{d^*_\alpha, \Delta^*_\alpha\}$ are those that minimise $\sum_\alpha N_\alpha H_\alpha$ subject to Lagrange multipliers enforcing the constraints Eqs. S18a–S18b.

Now the mean-field partition function can be written

$$Z_{MF} = \sum_{\{N_\alpha\}} \exp \left( -\beta \left[ NV^*(\bar{\phi}^t, \bar{\phi}^b) + \sum_\alpha (N_\alpha H_\alpha \{d^*_\alpha, \Delta^*_\alpha\} + k_B T N_\alpha \ln N_\alpha) \right] \right) \approx \sum_{\{N_\alpha\}} \exp (-\beta \bar{f}) \equiv \prod_\alpha \exp (-\beta N \bar{f}^*),$$ \hfill (S20)

where Stirling’s approximation has been used ($\ln N_\alpha \approx N_\alpha \ln N_\alpha - N_\alpha$), with a term proportional only to $N$ neglected.

The three constraints Eqs. S12a–S12c leave only one $N_\alpha$ to sum over. For this sum we perform a saddle-point approximation, which is equivalent to removing the sum and setting $\{N_\alpha\}$ to their values that minimise $\bar{f}$ subject to Eqs. S12a–S12c. This yields

$$Z_{MF} \approx \exp (-\beta N \bar{f}^*),$$ \hfill (S21)

where $\bar{f}^*$ is the minimised value of $\bar{f}$. Then, by Eq. S3 our desired free energy per site $f(\bar{\phi}^t, \bar{\phi}^b, \bar{d}, \Delta)$ is given by $\bar{f}^*$.

The steps described above can be summarised compactly by stating that the free energy $f(\bar{\phi}^t, \bar{\phi}^b, \bar{d}, \Delta)$ per site is given by minimising

$$\bar{f} N = \sum_\alpha (N_\alpha H_\alpha + k_B T N_\alpha \ln N_\alpha) - 2VN(\bar{\phi}^t - \bar{\phi}^b)^2 - 2VN(\bar{\phi}^t + \bar{\phi}^b - 1)^2,$$ \hfill (S22)
over \( \{ d_\alpha, \Delta_\alpha, N_\alpha \} \) subject to Lagrange multipliers enforcing Eqs. S18a and S12a–S12c as written in Eq. 2 of the main text.

The variables fixed in the minimisation procedure are

\[
\begin{align*}
\Delta_{SS} &= \bar{d} + \frac{\kappa \Delta_0}{2J + \kappa} (2 - \bar{\phi}_d - \bar{\phi}_b) , \\
\Delta_{UU} &= \bar{d} - \frac{\kappa \Delta_0}{2J + \kappa} (\bar{\phi}_d + \bar{\phi}_b) , \\
\Delta_{SU} &= \Delta_{US} = \bar{d} - \frac{\kappa \Delta_0}{2J + \kappa} (\bar{\phi}_d + \bar{\phi}_b - 1) , \\
\Delta_{SS} &= \Delta_{UU} = \bar{\sigma} - \frac{\kappa \Delta_0}{2B + \kappa} (\bar{\phi}_d - \bar{\phi}_b) , \\
\Delta_{SU} &= \Delta_{US} = \bar{\sigma} - \frac{\kappa \Delta_0}{2B + \kappa} (\bar{\phi}_d - \bar{\phi}_b + 1) , \\
N_{SS}/N &= A , \\
N_{UU}/N &= A + 1 - \bar{\phi}_d - \bar{\phi}_b , \\
N_{SU}/N &= -A + \bar{\phi}_d , \\
N_{US}/N &= -A + \bar{\phi}_b .
\end{align*}
\]  

We have defined

\[
A \equiv \frac{2\bar{\phi}_d \bar{\phi}_b}{\bar{\phi}^* \left(1 + \sqrt{1 + \frac{4\bar{\phi}_d \bar{\phi}_b (\exp[-2\beta \sigma])}{(\bar{\phi}^*)^2}}\right)} ,
\]

where

\[
\bar{\phi}^* \equiv \bar{\phi}_d + \bar{\phi}_b + \exp(-2\beta \sigma)(1 - \bar{\phi}_d - \bar{\phi}_b) ,
\]

and

\[
\sigma \equiv \frac{1}{2} \left( H_{SU} + H_{US} - H_{SS} - H_{UU} \right) = -\frac{\Delta_0^2 \kappa^2 (J - B)}{2(2J + \kappa)(2B + \kappa)} ,
\]

is the energy change per site for converting two R sites into two AR sites. The expected self-consistency requirements are fulfilled; for example, \( \bar{\sigma} \equiv \bar{\phi}_d + \bar{\phi}_b \) over the MF thickness variables \( \bar{\sigma} \equiv \bar{\phi}_d + \bar{\phi}_b \) and \( \bar{\phi}_d \rightarrow 1 \) (forcing all sites to be of SS type) leads to \( \Delta_{SS} \rightarrow d \).

To construct the coarse-grained free energy \( f(\bar{\phi}_d, \bar{\phi}_b, \bar{d}, \bar{\Sigma}) \) of the free energy, we insert Eqs. S23–S26 into Eq. S22. We find

\[
\begin{align*}
f(\bar{\phi}_d, \bar{\phi}_b, \bar{d}, \bar{\Sigma}) &= k_B T \left[ \ln A + (A + 1 - \bar{\phi}_d - \bar{\phi}_b) \ln (A + 1 - \bar{\phi}_d - \bar{\phi}_b) + (\bar{\phi}_d - A) \ln (\bar{\phi}_d - A) + (\bar{\phi}_b - A) \ln (\bar{\phi}_b - A) \right] \\
&\quad + \frac{1}{2\kappa} \left( (\bar{d} - d_0)^2 + \Delta_0 \left( (\bar{\phi}_d + \bar{\phi}_b - 1)(\bar{d} - \bar{d}) - (\bar{\phi}_d - \bar{\phi}_b) \bar{\Delta} + \frac{1}{2} \Delta_0 \right) \right) + \frac{1}{4} \bar{\Sigma}^2 (2B + \kappa) \\
&\quad + \frac{\kappa^2 \Delta_0^2}{2(2B + \kappa)(2J + \kappa)} \left[ (2A - 2\bar{\phi}_d \bar{\phi}_b) (J - B) - (\bar{\phi}_d + \bar{\phi}_b - \bar{\phi}_d^2 - \bar{\phi}_b^2) (J + B + \kappa) \right] \\
&\quad - 2V(\bar{\phi}_d - \bar{\phi}_b)^2 - 2V(\bar{\phi}_d + \bar{\phi}_b - 1)^2 ,
\end{align*}
\]

where \( \Delta_0 \equiv \ell_{s0} - \ell_{u0}, d_0 \equiv \ell_{s0} + \ell_{u0} \).

Upon further minimising \( f \) over the MF thickness variables \( \bar{d} \) and \( \bar{\Sigma} \), we obtain \( f^{\text{ann.}}(\bar{\phi}_d, \bar{\phi}_b) \), which determines local and global minima of the free energy (see e.g. Figs. 2, 3). The annealed thickness variables are

\[
\begin{align*}
\bar{d}^{\text{ann.}} &= \Delta_0 (\bar{\phi}_d + \bar{\phi}_b - 1) + d_0 , \\
\bar{\Sigma}^{\text{ann.}} &= \frac{\kappa \Delta_0 (\bar{\phi}_d + \bar{\phi}_b)}{2B + \kappa} ,
\end{align*}
\]


\[
\begin{align*}
\delta F_{G-L}^R &= \int d^2 r \left( \frac{1}{2\alpha^2} \left( (\delta \phi^t)^2 f^{R}_{\phi^t \phi^t} + (2\delta \bar{\phi}^t)^2 f^{R}_{\bar{\phi}^t \bar{\phi}^t} + 4\delta \bar{\phi}^t \delta \bar{\phi}^t f^{R}_{\bar{\phi}^t \bar{\phi}^t} \right) + 2V(\nabla \delta \bar{\phi}^t)^2 \right), \\
\delta F_{G-L}^{AR} &= \int d^2 r \left( \frac{1}{2\alpha^2} \left( (\delta \bar{\phi}^t)^2 f^{AR}_{\bar{\phi}^t \bar{\phi}^t} + (2\delta \bar{\phi}^t)^2 f^{AR}_{\bar{\phi}^t \bar{\phi}^t} + 4\delta \bar{\phi}^t \delta \bar{\phi}^t f^{AR}_{\bar{\phi}^t \bar{\phi}^t} \right) + 2V(\nabla \delta \bar{\phi}^t)^2 \right),
\end{align*}
\]

since \( \delta \bar{\phi} = 2\delta \bar{\phi}^t \). Subscripts indicate derivatives, evaluated at the mixed state, i.e. \( \bar{\phi}^t = 0.5 \), \( \bar{d} = d_0 \).

**B. Antiregistered mode**

For the antiregistered (AR) mode we have \( \delta \bar{\phi} = -\delta \bar{\phi}^t \) and \( \delta \bar{\phi} = -\delta \bar{\phi}^t \). These follow from the symmetry of the free energy under species interchange, implying a symmetry about \( \bar{\phi} = 0.5 \), \( \bar{\Delta} = 0 \). \( f^{AR}(\bar{\phi}^t, \bar{\Delta}) \) is \( f \) evaluated for \( \bar{\phi}_{AR}(\bar{\phi}^t) = 1 - \bar{\phi}^t \), \( d = d_0 \) and its derivatives are evaluated at the mixed state, i.e. \( \bar{\phi}^t = 0.5 \), \( \bar{\Delta} = 0 \). Hence,

\[
\delta F_{G-L}^{AR} = \int d^2 r \left( \frac{1}{2\alpha^2} \left( (\delta \bar{\phi}^t)^2 f^{AR}_{\bar{\phi}^t \bar{\phi}^t} + (2\delta \bar{\phi}^t)^2 f^{AR}_{\bar{\phi}^t \bar{\phi}^t} + 4\delta \bar{\phi}^t \delta \bar{\phi}^t f^{AR}_{\bar{\phi}^t \bar{\phi}^t} \right) + 2V(\nabla \delta \bar{\phi}^t)^2 \right),
\]

since \( \delta \bar{\phi} = 2\delta \bar{\phi}^t \).
C. Evolution of perturbations

The above can be rewritten in the form

$$\delta F^m_{G-L} = \frac{1}{2} \int d^2 r \left( \left( \frac{\delta \phi^m}{\delta \ell^m} \right) \cdot C^m \cdot \left( \frac{\delta \phi^m}{\delta \ell^m} \right) + \nabla \left( \frac{\delta \phi^m}{\delta \ell^m} \right) \cdot P^m \cdot \nabla \left( \frac{\delta \phi^m}{\delta \ell^m} \right) \right),$$  \hspace{1cm} (S34)

where \( m = R, AR \) and the matrices \( C^m \) and \( P^m \) respectively contain the bulk and gradient free energy terms:

$$a^2 C^R = \begin{bmatrix} f^R_{\phi \phi} & f^R_{R \phi} & 2 f^R_{R \phi} \\ 2 f^R_{R \phi} & 4 f^R_{R \phi} & 4 f^R_{R \phi} \\ \end{bmatrix} = \begin{bmatrix} \left( 4 k_B T \left[ e^{-\beta \sigma} + \beta \sigma + 1 \right] + \frac{2 \kappa^2 \Delta^2 (B + \kappa + J)}{(2 B + \kappa)(2 J + \kappa)} - 16 V \right) & -2 \kappa \Delta_0 \\ -2 \kappa \Delta_0 & 2 \kappa \end{bmatrix},$$  \hspace{1cm} (S35a)

$$a^2 C^{\text{AR}} = \begin{bmatrix} f^\text{AR}_{\phi \phi} & f^\text{AR}_{\phi \phi} & 2 f^\text{AR}_{\phi \phi} \\ 2 f^\text{AR}_{\phi \phi} & 4 f^\text{AR}_{\phi \phi} & 4 f^\text{AR}_{\phi \phi} \\ \end{bmatrix} = \begin{bmatrix} \left( 4 k_B T \left[ e^{\beta \sigma} - \beta \sigma + 1 \right] + \frac{2 \kappa^2 \Delta^2 (B + \kappa + J)}{(2 B + \kappa)(2 J + \kappa)} - 16 V \right) & -2 \kappa \Delta_0 \\ -2 \kappa \Delta_0 & 2 \kappa \end{bmatrix},$$  \hspace{1cm} (S35b)

$$P^R = \begin{bmatrix} 4 V & 0 \\ 0 & 4 J \end{bmatrix}, \quad P^{\text{AR}} = \begin{bmatrix} 4 V & 0 \\ 0 & 0 \end{bmatrix}.$$  \hspace{1cm} (S35c)

Since composition is conserved, it evolves \([S2]\) via

$$\frac{\partial \delta \phi^m}{\partial t} = M \nabla^2 \left( C^m_{11} \delta \phi^m + C^m_{12} \delta \ell^m - P^m_{11} \nabla^2 \delta \phi^m - P^m_{12} \nabla^2 \delta \ell^m \right),$$  \hspace{1cm} (S36)

where the mobility \( M \) sets the timescale.

We assume thickness to behave in a nonconserved fashion so that it evolves relaxationally \([S2]\), via

$$\frac{\partial \delta \ell^m}{\partial t} = -\eta \left( C_{21}^m \delta \phi^m + C_{22}^m \delta \ell^m - P_{21}^m \nabla^2 \delta \phi^m - P_{22}^m \nabla^2 \delta \ell^m \right),$$  \hspace{1cm} (S37)

where the mobility \( \eta \) incorporates frictional forces involved in length stretching and compression of amphiphiles (in principle it can acquire wavenumber dependence via coupling to the conserved solvent flow).

In Fourier space, the coupled evolution equations are

$$\frac{\partial}{\partial t} \left( \frac{\delta \phi_{\ell \ell}^m}{\delta q^m} \right) = -M(q) \cdot \left( C^m + q^2 P^m \right) \cdot \left( \frac{\delta \phi^m}{\delta q^m} \right),$$  \hspace{1cm} (S38)

where

$$M(q) = \begin{bmatrix} M q^2 & 0 \\ 0 & M \epsilon \end{bmatrix}.$$  \hspace{1cm} (S39)

The dimensionless parameter \( \epsilon \equiv \eta/M \) controls how ‘fast’ the thickness relaxation is relative to diffusion. Instabilities of the R or AR mode correspond to a negative eigenvalue of their \( L^m \). Their wavenumber dependent growth rates are given by \( \omega^m(q) = -\lambda^m \) where \( \lambda^m \) is the eigenvalue for the eigenmode of \( L^m \). Maximising \( \omega^m(q) \) over \( q \) yields \( \omega^m_{\text{max}} \), the peak growth rate of the given mode (R or AR).

The blue and red colours in Fig. 4 are obtained by first calculating \( \omega^R_{\text{max}} \) for the R and AR modes. Then the difference \( \Delta \omega \equiv \omega^R_{\text{max}} - \omega^m_{\text{max}} \) is plotted as the background of Fig. 4. If a given mode \( m \) has a negative peak growth rate (i.e. is not unstable) then its \( \omega^m_{\text{max}} \) is set to zero. Thus \( \Delta \omega = 0 \) (white) is ambiguous; either i) both modes are stable or ii) both are unstable but with equal peak growth rates. This ambiguity is easily resolved by referring to the instability lines when interpreting the plot, since if mode \( m \)’s peak growth rate is zero then we must be outside the instability region of mode \( m \). Note that the ranges of the colour scales in Fig. 4 are asymmetric.

To model the physically likely scenario we have used \( \epsilon = 100 \), since any frictional drag involved in stretching
should be far less than that for lateral diffusion [S2, S3]. This value is close to ‘saturation’, i.e. the composition relaxation is the limiting timescale and significant further increases in \( \epsilon \) have only marginal quantitative effects on \( \omega_m(q) \). Therefore the conclusions drawn from the colours in Fig. 4 are independent of \( \epsilon \) in the expected physical regime. Even if the opposite regime is assumed (\( \epsilon = 0.1 \)), the values of \( \omega_m(q) \) change but the key feature of the \( \Delta \omega \) landscape – which mode is fastest – is not strongly affected.

### III. PHYSICAL MEANING OF PARAMETERS

For comparison with phospholipids, we set the lattice spacing \( a \approx 0.8 \text{ nm} \), corresponding to an area per lipid of 0.64 nm\(^2\) [S4].

#### A. Stretching modulus

The stretching and compression of a bilayer of amphiphiles is measured experimentally via the area stretching modulus \( \kappa_A \), with a free energy given by

\[
G_{\kappa_A}^{\text{bilayer}} = \int d^2r \frac{\kappa_A}{2} \left( \frac{\delta A}{A_0} \right)^2, \quad (S40)
\]

where \( \delta A \) represents an area difference relative to the equilibrium area \( A_0 \). In the continuum representation of the lattice model here the stretching free energy for an individual leaflet of the bilayer is given by

\[
G_{\kappa}^{\text{leaflet}} = \int d^2r \frac{\kappa}{a^2} (\delta \ell)^2, \quad (S41)
\]

where \( a^2 \) is the lattice site area and \( \delta \ell \) is an amphiphile length difference relative to an equilibrium length \( \ell_0 \). Assuming that the volume \( v = A \ell \) remains constant upon stretching/compression, \( A \delta \ell = -\ell \delta A \), we have

\[
G_{\kappa}^{\text{leaflet}} = \int d^2r \frac{\ell_0 \kappa}{a^2} \left( \frac{\delta A}{A_0} \right)^2. \quad (S42)
\]

Noting that Eq. (S40) describes the area stretching energy for the whole bilayer, and assuming the energy to be distributed evenly between the two leaflets, we can write

\[
G_{\kappa}^{\text{leaflet}} = \frac{1}{2} G_{\kappa_A}^{\text{bilayer}}. \quad (S43)
\]

Identifying the lattice site area \( a^2 \) with the equilibrium area per amphiphile \( A_0 \) gives the correspondence

\[
\kappa = \frac{A_0}{2 \ell_0^2} \kappa_A, \quad (S44)
\]

where \( \ell_0 \) is a representative value for the equilibrium length of a real amphiphile. For typical values \( \ell_0 = 2 \text{ nm} \) and \( A_0 = 0.64 \text{ nm}^2 \) for phospholipid leaflets, the value \( \kappa = 3 a^2 k_B T \) used in this work corresponds to \( \kappa_A \approx 40 a^2 k_B T \approx 60 k_B T \text{ nm}^{-2} \), in the range for lipid bilayers at 300 K [S2, S3, S4].

#### B. Indirect coupling

The indirect coupling parameter \( J \) quantifies the penalty for mismatch in the total hydrophobic thickness between neighbouring lattice sites, arising from hydrophobic surface tension. We take a fiducial value \( J \approx 0.8 k_B T \text{ nm}^{-2} \), approximately that estimated in Ref. [S3] as a surface tension for hydrocarbon tails in contact with the watery headgroups of phospholipids. This gives \( J \approx 0.5 a^{-2} k_B T \) for the lattice model, so for the mean-field parameter \( J \approx 2 a^{-2} k_B T \). Increasing \( J \) (Fig. 4) can be thought of as increasing hydrophobic mismatch/hydrophobicity.

#### C. Direct coupling

The direct coupling parameter \( B \) plays a similar role to the inter-leaflet mismatch free energy \( \gamma \) estimated in the literature. We can define an effective \( \gamma \) (that shown in Fig. 4) by considering an isolated AR site and minimising its energy arising from stretching (\( \kappa \)) and direct coupling (\( B \)) energies over the top and bottom amphiphile lengths, where the reference state is an isolated R site which experiences zero direct coupling energy. This microscopic energy per AR site is

\[
\gamma a^2 = \frac{\Delta_0^2 \kappa B}{2(\kappa + 2B)}, \quad (S45)
\]

in terms of which

\[
B = \frac{2\gamma a^2 \kappa}{\kappa \Delta_0^2 - 4\gamma a^2}. \quad (S46)
\]

For example, the value \( \gamma \approx 0.15 k_B T \text{ nm}^{-2} \) estimated in Ref. [S7] is, in model units, \( \gamma \approx 0.1 a^{-2} k_B T \). Assuming \( \Delta_0 = 1 a \) and \( \kappa = 3 a^{-2} k_B T \), this gives \( B \approx 0.23 a^{-2} k_B T \). In comparison to the other parameters, though, \( \gamma \) is poorly understood. Ref. [S8] estimates an order of magnitude lower (\( \gamma \sim 0.01 a^{-2} k_B T \), so that \( B \sim 0.02 a^{-2} k_B T \)), and finds that the method used to extract \( \gamma \) in simulation [S7] is inaccurate, since it assumes larger characteristic fluctuations than were measured. On the other hand, Ref. [S9] finds that the effective \( \gamma \) measured while artificially pulling domains out of registration depends strongly on mismatch area, and proposes a role for membrane curvature, which we have not studied.

#### D. Interpretation of \( \gamma \)

There are subtleties in defining the mismatch free energy per area \( \gamma \). We have defined it ‘microscopically’ in Eq. (S45) as the direct coupling energy density for an antiregistered site. It is possible instead to construct a ‘macroscopic’ definition by comparing the free energies
of antiregistered and registered domains
\[ \gamma_{\text{macro}} \equiv \lim_{A \to \infty} \frac{1}{A} \left( G^{\text{anti-reg}}(A) - G^{\text{reg}}(A) \right), \quad (S47) \]

where \( G^{\text{(anti)reg}}(A) \) is the free energy of an (anti)registered domain of area \( A \). We have included the limit \( A \to \infty \) to emphasise that any boundary contributions to the free energies are ignored \[S8\] \[S9\] \[S10\]. See for example Ref. \[S8\], in which \( \gamma_{\text{macro}} \) is computed theoretically by comparing the free energies of an antiregistered and registered arrangement of domains within a molecular mean-field theory, the domains being assumed large enough that contributions from their boundaries can be neglected. It is important to note that any effects of hydrophobic mismatch energy at the edges of registered domains (incorporated in our Ginzburg-Landau analysis via the \( J \) term of \( f_{\text{grad}} \)) cannot be properly captured by \( \gamma \) or \( \gamma_{\text{macro}} \), since these describe only energies that scale as the domain area.

In the well segregated limit such that an anti(registered) domain contains purely pairwise (anti)registered sites, the definition Eq. \[S47\] becomes equivalent to Eq. \[S45\]. Near this limit, within our model \( \gamma_{\text{macro}} \approx \gamma \), because the dominant contribution to the free energy difference in Eq. \[S47\] will be from the direct coupling energy experienced by AR sites (Eq. \[S45\]), while contributions associated with the remnant fraction of pairwise R sites in the antiregistered demixed phase (and vice versa) will be small. Thus, for example, the free energy difference between the R and AR minima of \( f^{\text{ann.}} \) (Fig. 2) is similar to the value of \( \gamma \) quoted for that state point on Fig. 4e, calculated with Eq. \[S45\].

In general, however, Eq. \[S47\] requires specification of the compositions of the R and AR phases whose free energies are to be compared, and Fig. 3 shows us that the leaflet compositions in the AR phases generally differ from the those in the R phases. Therefore, the assumption \[S8\] that the relevant AR configuration for comparison is that obtained by re-arranging the domains from the R configuration, without altering their compositions, is incorrect. It may be suitable for describing small fluctuations into AR at the boundary of a large R domain (as was the purpose in Ref. \[S8\]), but only if one assumes that spatial fluctuations of the domain boundaries out of registration are not also accompanied by compositional fluctuations of the domains in each leaflet.

In some situations, even an approximate equality between \( \gamma \) and \( \gamma_{\text{macro}} \) breaks down due to ambiguity in implementing the macroscopic definition. Taking as given that R demixed phases exist in the free energy landscape, one might naturally assume that we should take the metastable AR phases in the free energy landscape for comparison. However, in Fig. 3 (top pane), R demixed minima exist but no metastable AR demixed minima exist – there is thus no thermodynamically (meta)stable AR phase to compare against the R phase in Eq. \[S47\]. In another case, in a small region of Fig. 4b the antiregistered phases are equilibrium. R demixed phases exist for comparison but are of higher free energy, which under Eq. \[S47\] would imply a negative value of \( \gamma_{\text{macro}} \), although the per-site \( \gamma \) defined by Eq. \[S45\] is positive.

Hence, it is clear that describing inter-leaflet coupling is complex, both in terms of specifying the relevant bulk free energy and in terms of the domain size-dependent competition of edge and area energies. This latter aspect in particular, and its role in nucleation kinetics of domain registration, will be further studied in an upcoming publication. In relation to the present discussion, it is unclear precisely which coupling or combination of couplings is being measured in molecular simulation studies of inter-leaflet coupling \[S7\], where the probability of fluctuations into antiregistration is monitored and fit to a Boltzmann distribution. These fluctuations may be subject to effects related to hydrophobic mismatch and composition dependence as discussed above, so that even if the approach of measuring fluctuations is essentially correct (challenged in Ref. \[S8\]), it is likely that energies additional to that described by \( \gamma \) are at work. In summary, much further work is required in defining, measuring, and studying the implications of the competing forms of inter-leaflet coupling.

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