Dominance metric in multi-component binary phase equilibria

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Binary phase separation with conserved order parameters is a generic thermodynamic process that arises in different contexts. One system of great fundamental and practical interest is the condensation of bio-molecules, where the order parameters are concentrations of solutes, and the condensed phase typically contains many different components of diverse properties. In such systems, each solute species corresponds to one dimension in the full phase space and a major challenge in the field is to rigorously interpret and quantify the multi-component character of any given phase separation system. Here, we formulate a dominance metric D^α for each solute α in a multi-component system, and show that it is easily accessible in experiments and relates to the underlying thermodynamics and the phase space. We complement the theoretical work with experimental data, paving the way for future investigations into multi-component systems.

The general principle of liquid-liquid binary phase separation is simple: starting from a single mixed solution state, the solutes de-mix to form two compartments with distinct compositions to lower the system's total free energy, and this free energy change is denoted by Δf. The two compartments formed have equal chemical potentials and osmotic pressure, as required by phase equilibria [1]. This process is the theoretical underpinning of bio-molecular condensate formation [2–4], which has been shown to play important roles in cellular physiology [5–9] and also diseases [10, 11]. Experimentally, the phase-separating macromolecule - typically a protein - is tagged by a florescent dye, and the protein concentration can be measured through fluorescence microscopy. As a result, it normally appears that the system forms a protein-rich dense phase and a protein-poor dilute phase, and the 1-D picture is often assumed [12–15]. This, however, is an over-simplification as many more solute dimensions are ignored, and the dilute and dense phases can differ not only in macromolecule concentration but also in pH [16] and salt concentrations [17], so important mechanistic information can be lost due to this oversight. Notably, the macromolecule polyethylene glycol (PEG) has traditionally been used by the bio-molecular condensate field as an inert crowder [11, 18] but it is increasingly clear that PEG is acting as an associative linker that partitions inside condensates [19–21], instead of staying in the dilute phase to exert an osmotic pressure.

A major challenge thus arises: how can one define, quantify, and measure, the multi-component character of such systems? Furthermore, solute species present in experiments have very different properties, for instance the molecular weight of a typical protein is of the order 100 kilodalton, while that of salt is of the order 100 dalton - how can these species be compared on an equal footing? This challenge calls for a dimensionless metric that satisfies the following conditions:

1. It can be experimentally measured;
2. It is defined for every solute in the same way, irrespective of their molecular details;
3. It reflects the underlying thermodynamics.

Surprisingly, a metric that satisfies conditions 1 and 2 can be rather easily constructed. Denoting the total concentrations of solutes to be ϕ^α where $\alpha = 1, 2, \ldots, N$, and suppose that the dilute phase concentration of solute 1, denoted as ψ^1_-, can be measured. A series of experimental samples can be prepared with varying ϕ^1 while keeping all other ϕ's constant and measuring ψ^1_-. This corresponds to traversing the phase space in a line parallel to the ϕ^1 axis and we term this 'homotypic line-scan' (Figure 1a). We take experimental data on phase separation of the optoG3BP1 protein in cells [22] where such a response curve is measured, treating the protein as solute 1 (Figure 1b). Denoting the gradient of the response $\frac{\partial \psi^1_-}{\partial \phi^1}$ as R^1_1, we trivially have $R^1_1 = 1$ outside the phase-separated region, and the response becomes modulated beyond some critical value ϕ^1_c. In an ideal 1-D system, for $\phi^1 > \phi^1_\mathrm{c}$ we expect $R^1_1 = 0$, while because of the multi-component nature of the system [22] we have $R^1_1 > 0$ for optoG3BP1. We denote this deviation of R^1_1 at $\phi^1 > \phi^1_\mathrm{c}$ from 1 as D^1, with the index 1 implying a similar metric can in principle be measured for any other solutes. D^1 is defined as $D^1 \equiv 1 - R^1_1$, or more generally for a solute with index α,

$$D^\alpha \equiv 1 - R^\alpha_\alpha \tag{1}$$

where repeated greek indices are not summed in this manuscript. Using a 2-segment linear fit for optoG3BP1

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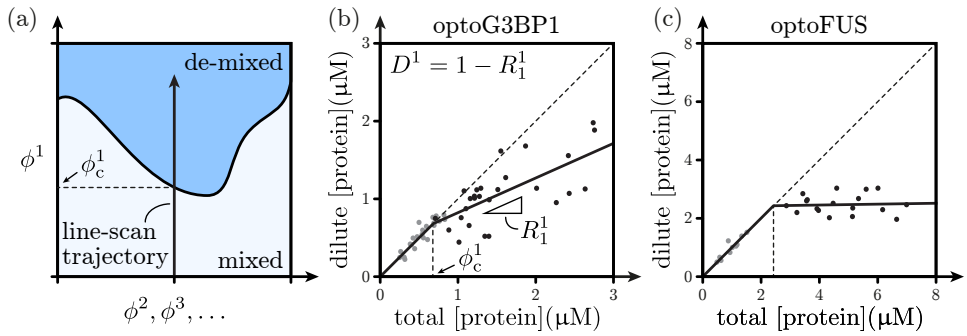

FIG. 1. **Dilute phase homotypic responses naturally define the dominance metric.** (a) Illustration of the line-scan sampling method in a generic phase space. The dilute phase solute 1 concentration increases until the sampling enters the phase-separated region at $\phi^1 = \phi^1_c$, after which the increase becomes modulated by phase separation. (b) The dilute phase response of optoG3BP1 [22] exhibits multi-component character, with $D^{(1)} = 1 - R^1_1 = 0.55 \pm 0.14$. (c) In the optoFUS system [23] the protein has much stronger single-component character. Scatter plot is data with black dots being phase separated and grey dots homogeneous. Diagonal dashed line is expected response without phase separation, and solid line is a 2-segment linear fit.

data we obtain $D^1 = 0.55 \pm 0.14$. In contrast, in another system involving the protein optoFUS [23], the protein exhibits much more prominent single-component character (Figure 1c) and the dominance metric of optoFUS is $D^1 = 0.98 \pm 0.07$. This is consistent with the observation that FUS phase separates easily on its own [13, 24] and can be less reliant on other components compared to G3BP1, where other macromolecules such as RNA are typically added to G3BP1 solution to trigger phase separation [8, 25].

The origin of D^α defined using Eq. (1) can be traced to the phase space. In a generic N-D system, each point on the dilute phase boundary ψ^α_- is connected to another point on the dense phase boundary ψ^α_+, via a tie line vector $k^\alpha \equiv \psi^\alpha_+ - \psi^\alpha_-$. For a sample point ϕ^α within the phase separated region, the dilute and dense phase compositions that arise at equilibrium satisfy the condition that ϕ^α, ψ^α_-, and ψ^α_+ all lie on the same tie line, as required by mass conservation. The volume of the dense phase relative to the whole system is denoted as v, so $0 \leq v \leq 1$ and v represents the position of ϕ^α on the tie line k^α. The mass conservation condition is expressed as $\phi^\alpha = (1-v)\psi^\alpha_- + v\psi^\alpha_+$. Furthermore, the dilute phase boundary is an $(N-1)$-D surface and we use n_α to denote the vector normal to this surface. This construction of the phase space is entirely geometrical and the only assumption is that two phases co-exist at equilibrium (Figure 2a). It is easily shown via mass conservation that changes in ψ^α_- in response to changes in ϕ^α (Figure 2b), $\frac{\partial \psi^\alpha_-}{\partial \phi^\beta} \equiv R^\alpha_\beta$, can be expressed as (SI section I)

$$R^\alpha_\beta = \delta^\alpha_\beta - \frac{n_\beta}{n_\alpha}\left(\frac{n_\alpha k^\alpha}{\sum_{\gamma=1}^N n_\gamma k^\gamma}\right) = \delta^\alpha_\beta - \frac{n_\beta}{n_\alpha} D^\alpha \tag{2}$$

under the assumption that v is small or the tie lines are locally parallel. δ^α_β is the Kronecker delta and in the second equality we observe that by setting $\beta = \alpha$ and noting Eq. (1), the proposed dominance metric can be expressed as the geometrical quantity

$$D^\alpha \equiv \frac{n_\alpha k^\alpha}{\sum_{\gamma=1}^N n_\gamma k^\gamma} \tag{3}$$

satisfying the condition that the dominance metric, if evaluated for all solutes, should sum to unity:

$$\sum_{\alpha=1}^N D^\alpha = 1. \tag{4}$$

Intuitively, if D^α for a solute α is found to be close to 1 in a system, this implies $D^\beta \approx 0$ for $\beta \neq \alpha$ and the system is effectively single-component. On the other hand, for a multi-component system, we expect D^α to be in the range of (0,1) for two or more solutes. The D^α metric can thus serve as a way to quantify the multi-component character of solutes in a system. It remains to show that the third condition for a good dominance metric is satisfied by D^α: that it reflects the thermodynamics of the phase separation process.

As outlined at the very beginning, the thermodynamic driving force is Δf, the free energy change of the whole system after phase separation (Figure 2c). Given a free energy density $f(\phi)$ of a system as a function of its composition ϕ^α, we can show that by limiting the dense phase volume fraction v to be small, equivalent to probing regions close to the dilute phase boundary, Δf is given by (SI section II)

$$\begin{aligned}\Delta f &= -\frac{1}{2}\sum_{\alpha=1}^N\sum_{\beta=1}^N vk^\alpha vk^\beta \partial_\alpha\partial_\beta f(\psi_-) + \mathcal{O}[(vk)^3] \\ &= -\frac{1}{2}\sum_{\alpha=1}^N\sum_{\beta=1}^N vk^\alpha vk^\beta \partial_\beta \mu_\alpha(\psi_-) + \mathcal{O}[(vk)^3]\end{aligned} \tag{5}$$

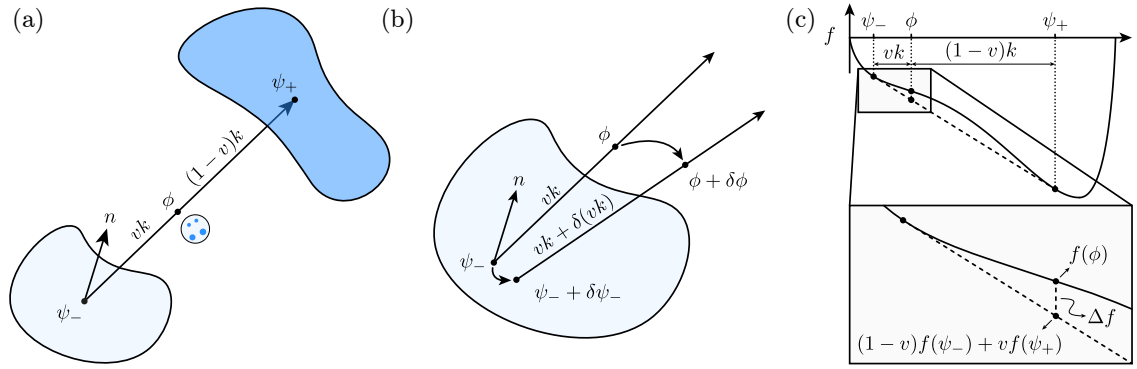

FIG. 2. **Geometrical construction of the phase space and its relation to thermodynamics.** (a) The full N-D phase space consists of dilute (light blue) and dense (dark blue) phase binodal boundaries, tie lines k^α connecting them, and dilute boundary surface normals n_α. (b) Experiments can probe the high-dimensional space by sampling two phase-separated points (ϕ^α and $\phi^\alpha + \delta\phi^\alpha$) and observing the response of the dilute phase $\delta\psi_-^\alpha$. (c) The free energy before and after phase separation is $f(\phi)$ and $(1-v)f(\psi_-) + vf(\psi_+)$ respectively, and a 1-D representation is used here but this is generalisable to any number of dimensions.

which is negative given all eigenvalues of the Hessian $\partial_\alpha\partial_\beta f$ are positive, since the system is locally stable near the binodal boundary. In the second line we used the expression for chemical potential $\mu_\alpha(\phi) = \partial_\alpha f(\phi)$. Since $vk^\alpha = \phi^\alpha - \psi_-^\alpha$ represents the phase space displacement of the total composition from the dilute composition, the sum over β can be viewed as computing the change in chemical potential of solute α under phase separation. We define the individual term

$$\Delta f^\alpha \equiv -\frac{1}{2} vk^\alpha \sum_{\beta=1}^{N} vk^\beta \partial_\beta \mu_\alpha(\psi_-) \tag{6}$$

such that if we form the ratio $\frac{\Delta f^\alpha}{\Delta f}$ we can write

$$\sum_\alpha \frac{\Delta f^\alpha}{\Delta f} = 1, \tag{7}$$

which is of a very similar form to Eq. (4). Indeed, by perturbing the equilibrium compositions we can show that $n_\alpha \propto \sum_{\beta=1}^{N} k^\beta \partial_\alpha\partial_\beta f(\psi_-)$ (SI section III) and as such, the equivalence of D^α and $\frac{\Delta f^\alpha}{\Delta f}$ can be established:

$$D^\alpha = \frac{\sum_{\beta=1} k^\alpha k^\beta \partial_\alpha\partial_\beta f(\psi_-)}{\sum_{\gamma=1}^{N}\sum_{\sigma=1}^{N} k^\gamma k^\sigma \partial_\gamma\partial_\sigma f(\psi_-)} = \frac{\Delta f^\alpha}{\Delta f}. \tag{8}$$

This validates D^α as a metric grounded on thermodynamics. The physical interpretation of D^α is then the amount of stabilisation free energy contributed by solute α relative to that by all solutes, and this gives quantitative meaning to the assessment of a system being single or multi-component. To further establish D^α as a valid dominance metric, we investigate how D^α responds to modulations of phase separation.

Poly(A)-RNA phase separates in the presence of PEG and high salt concentrations [21, 26] and we perform homotypic line-scans with 4% PEG (molecular weight 10 kilodaltons), and 800mM or 850mM of KCl (SI section IV). Dilute phase poly(A) concentrations are measured, so we use index 1 to denote poly(A) and index 2 for KCl, and we have $\Delta\phi^2 = 50$mM. Line-scan data (Figure 3a) shows a smaller R_1^1 after phase-separation at higher KCl, leading to an increase in D^1 from 0.49 ± 0.05 to 0.66 ± 0.04. The increase in poly(A) dominance at higher KCl can be explained by the increased screening of negative backbone charges on poly(A), facilitating poly(A)-poly(A) interaction and rendering other interactions, for instance poly(A)-PEG, less significant. An interesting observation is that the onset of phase-separation shifts to a lower poly(A) concentration when salt concentration is increased, with $\Delta\phi_c^1 = -0.54 \pm 0.12$g/l. This can be interpreted as an increase in overall phase-separation propensity,

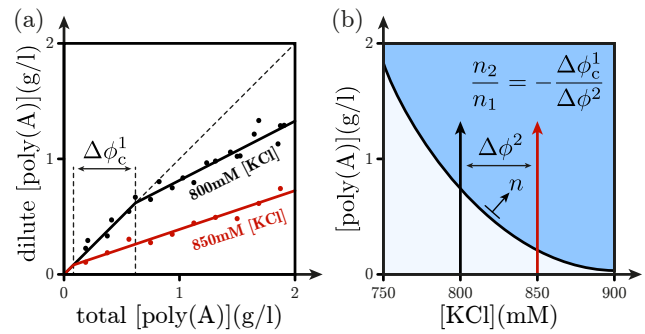

FIG. 3. **Modulation effect of KCl on poly(A) phase separation.** (a) Line-scan data for phase-separation of poly(A)-RNA in the presence of PEG and KCl. At higher [KCl] the increased charge screening allows inter-RNA interaction to be more favourable, leading to an increase in D^1 when [KCl] is increased. (b) Phase space illustration of the poly(A)-KCl cross-section.

consistent with the picture that poly(A)-poly(A) repulsion weakens with more salt. By combining $\Delta\phi^2$ with $\Delta\phi_c^1$ we can deduce the ratio of surface normals $\frac{n_2}{n_1}$ in the poly(A)-KCl plane (Figure 3b). This gives $\frac{n_2}{n_1} = -\frac{\Delta\phi_c^1}{\Delta\phi^2} = 10.8 \pm 2.4 \frac{\text{g/l}}{\text{M}}$. By combining this with D^1, we can further estimate the ratio of tie line components, $\frac{k^1}{k^2}$, even though we have not explicitly measured any KCl concentration.

We construct the quantity $K_2^1 \equiv \frac{n_2}{n_1}\frac{D^1}{1-D^1}$, and using Eq. (3) we arrive at (SI section V)

$$K_2^1 \equiv \frac{n_2}{n_1}\frac{D^1}{1-D^1} = \frac{k^1}{k^2}\left[\frac{1}{1+\sum_{\alpha=3}^{N} D^\alpha/D^2}\right] \tag{9}$$

and to leading order, K_2^1 provides an approximation of the ratio of tie line components $\frac{k^1}{k^2}$. For the poly(A) system, we use the average of the D^1 and arrive at an approximate tie line component ratio of 9.4 ± 1.8g/l of poly(A) per 1M of KCl, so KCl partitions into poly(A) condensates. This is in essence the tie line estimation method used in [19] (SI section V). Terms in the square bracket of Eq. (9) represent the deviation of K_2^1 from $\frac{k^1}{k^2}$, and in a true 2-component system we expect $D^\alpha = 0$ for $\alpha = 3, 4, \ldots, N$, from which we have exactly $K_2^1 = \frac{k^1}{k^2}$. By assuming the dominance of a typical solute falls within the $(0, 1)$ range the corrections do not change the sign of K_2^1, so K_2^1 provides a easy way of assessing whether the solute 2 is partitioning into $\left(\frac{k^1}{k^2} > 0\right)$ or out of $\left(\frac{k^1}{k^2} < 0\right)$ condensates. Notably, this partitioning information becomes accessible without directly measuring the solute 2, giving our approach a high degree of flexibility. An important corollary arising from Eq. (9) is that given a phase-separation solute 1 with $0 < D^1 < 1$, the sign of K_2^1 is entirely determined by the sign of $\frac{n_2}{n_1}$, and thus the sign of $\frac{k^1}{k^2}$ can be deduced by simply observing the effect of the solute 2 on condensates. To put this in practical terms, if solute 2 dissolves condensates $\left(\frac{n_2}{n_1} < 0\right)$ it partitions out of condensates $\left(\frac{k^1}{k^2} < 0\right)$ and vice versa, provided that D^1 falls within 0 and 1. This makes intuitive sense since it suggests that condensate-favouring modulators partition into condensates while condensate-disfavouring modulators are excluded from condensates. It is important however to point out here that D^1 can fall outside the 0 and 1 range, and we do not preclude this possibility even though all D^1 values cited in this work satisfies the 'normal' solute condition $0 < D^1 < 1$.

A subtle point here in calculating the tie line component ratio involving KCl is that the dissociation of the salt into K^+ and Cl^- means that KCl is not a single solute, and the line-scan is not traversing parallel to one axis but at an angle between two axes corresponding to K^+ and Cl^-, so mixing them is equivalent to performing a rotation of the two axes. This does not affect $D^1 = \frac{n_1 k^1}{\sum_{\gamma=1}^{N} n_\gamma k^\gamma}$ since the denominator is coordinate-independent and the numerator is not affected. Furthermore, since k^α and ϕ^α transform in the same way the measured K_2^1 represents the summed partitioning of K^+ and Cl^-. Converting between units and mixing solutes are both examples of general coordinate transformations in the phase space, and the geometrical nature of the theoretical construction allows these to be treated rigorously.

The discussion so far has focused on situations where the dilute phase concentrations are accessible for only a single solute, based on typical experimental constraints. In the case where multiple solutes can be measured post phase separation, not only can their D^α and ratios of k^α be worked out, entries in the Hessian $\partial_\alpha\partial_\beta f(\psi_-)$ can be obtained as well, which correspond to interaction energies of solutes in the dilute phase. This is based on the observation that $n_\alpha \propto \sum_{\beta=1}^{N} k^\beta \partial_\alpha\partial_\beta f(\psi_-)$, and n_α is relatively accessible via high-throughput platforms such as PhaseScan [27]. To illustrate the point, suppose $\partial_\alpha\partial_\beta f(\psi_-)$ consists of two terms: a constant, symmetric interaction energy matrix $\chi_{\alpha\beta}$ and a diagonal entropic term diag $\left(\frac{1}{L^1\psi_-^1}, \frac{1}{L^2\psi_-^2}, \ldots, \frac{1}{L^N\psi_-^N}\right)$, where L^α are sizes of the solute molecules. In doing so the free energy from translational entropy of solute α is assumed to be $\frac{\psi_-^\alpha}{L^\alpha}\ln\psi_-^\alpha$. Treating the interaction matrix and sizes L^α as unknowns, we have $N(N+1)/2 + N = \frac{1}{2}N^2 + \frac{3}{2}N$ fitting parameters. On the other hand, at each point ψ_-^α, we can form $N-1$ linearly independent equations using ratios of n_α's, so for an N-component system $\sim \frac{1}{2}N$ different sampling points ψ_-^α are needed. Practical implementation of this procedure can however be difficult, given the possibly large number of relevant solutes, but the prospect of obtaining $\chi_{\alpha\beta}$ in the dilute solution not only helps with understanding phase separation but also illuminates protein interactions in general, and can be a highly promising direction for further research.

To summarise, we construct an experimentally-accessible, dimensionless metric D^α to quantify the dominance of solutes in a binary phase separation system. The origin of D^α can be traced back to the thermodynamics, and combined with the dilute phase boundary surface normal n_α we enable tie line component ratios to be easily determined without the need to measure all solute concentrations. We outline the data processing method using published results and illustrate the utility of D^1 in a practical poly(A) system, paving the way for systematic studies of phase separation in the future.

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