From Blobs to Rods
Navigating Liquid-Liquid Phase Separation

Advanced Crystallisation Workshop

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Laboratory Services

Chemistry, Manufacturing and Control

Radiolabelled Sciences

Clinical Development

Biologics

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- **Why Does LLPS Occur?**

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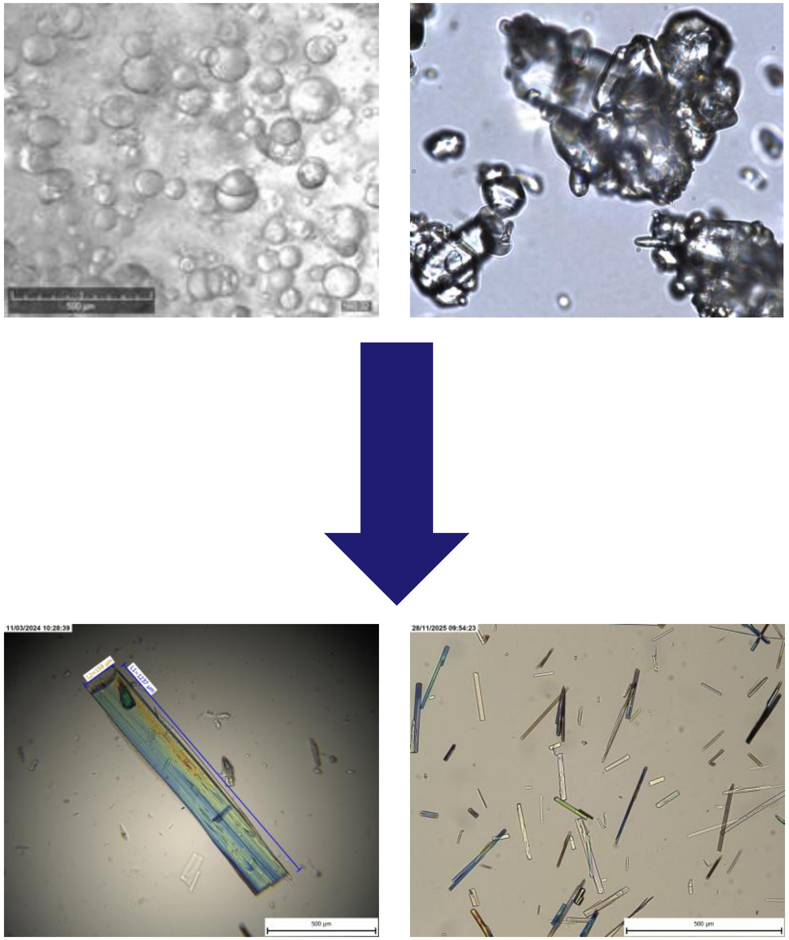
- **Triangular Phase Diagrams**

Avoiding LLPS

- **Seeding and Supersaturation Control**
- **Other Strategies**



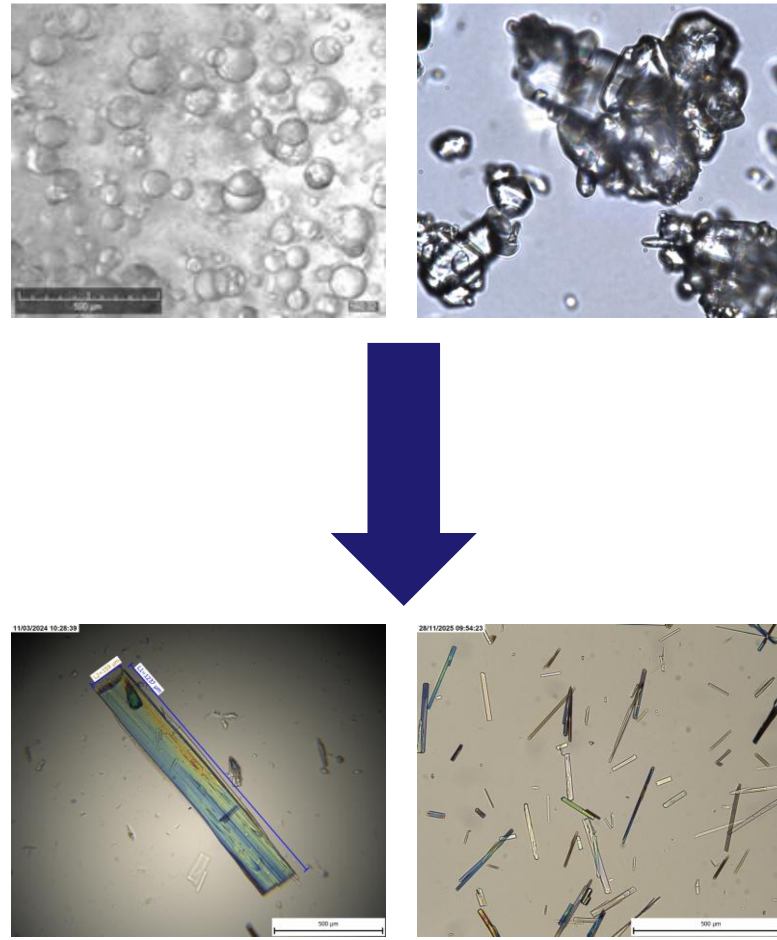
Glossary

- **Gibbs free energy of mixing:** the energy governing the process of mixing of phases (temperature, pressure, enthalpy of mixing and entropy of mixing are considered). The Gibbs free energy is the maximum amount of non-expansion of mixing. Note: When the free energy is negative, a spontaneous reaction occurs. When the free energy is positive, a nonspontaneous reaction occurs. When the free energy is equal to zero, the system has achieved a state of equilibrium.

- **Spinodal Decomposition:** a mechanism by which a single thermodynamic phase spontaneously separates into two phases (without nucleation).

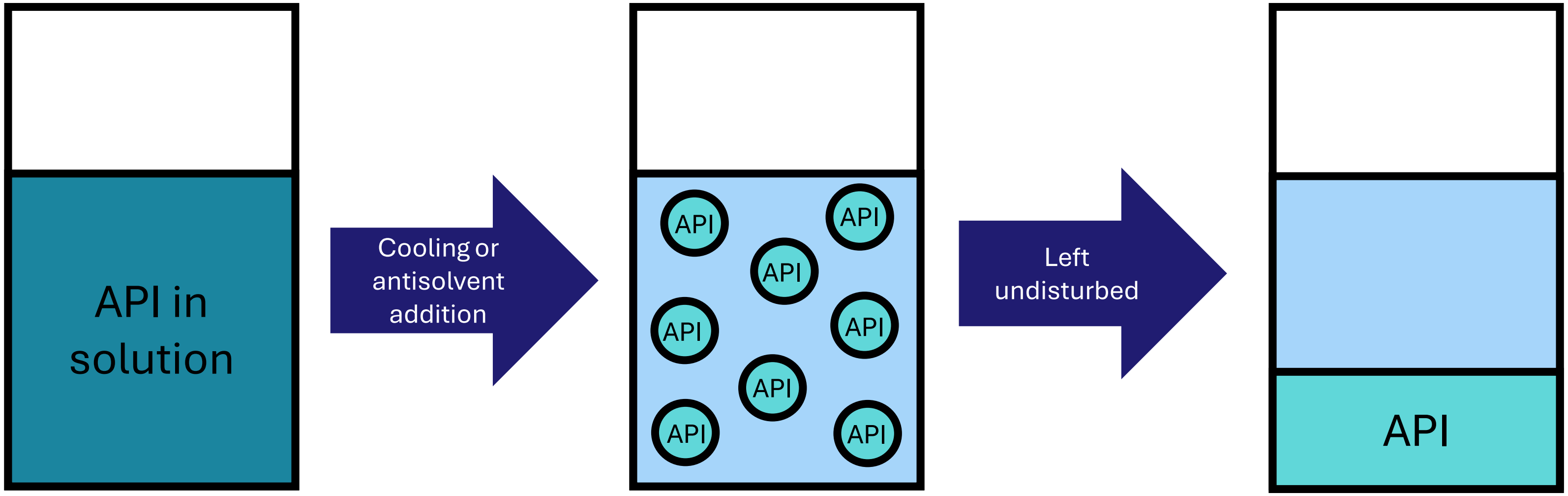
- **Miscibility gap:** areas in phase diagram representing the oiling out region/zone. These areas depend on temperature, pressure and system composition (mole fractions).

Contents



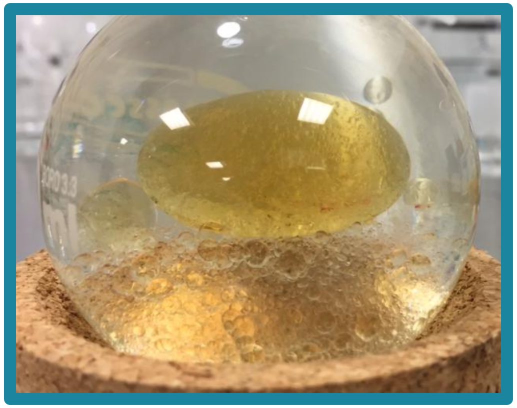
What is Liquid-Liquid Phase Separation?

- Liquid-Liquid phase separation (LLPS) ‘**oiling out**’ refers to the emergence of oil like droplets in solutions.
- These oil droplets are rich in solute (active pharmaceutical ingredient, API) and can separate into a distinct layer if left undisturbed.
- LLPS can be triggered by cooling or antisolvent addition.

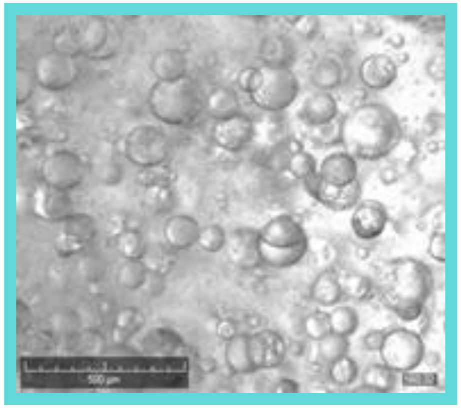


How Can We Spot LLPS?

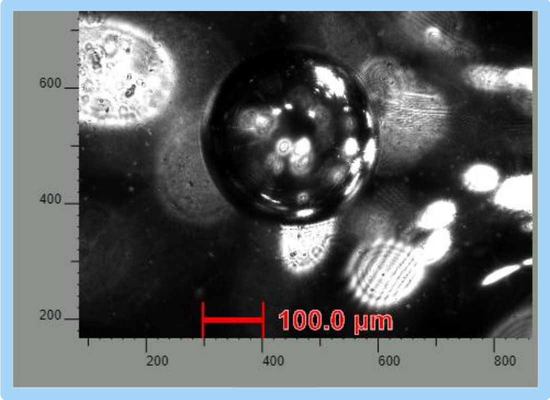
Visual LLPS



Crystalline



Blaze Probe

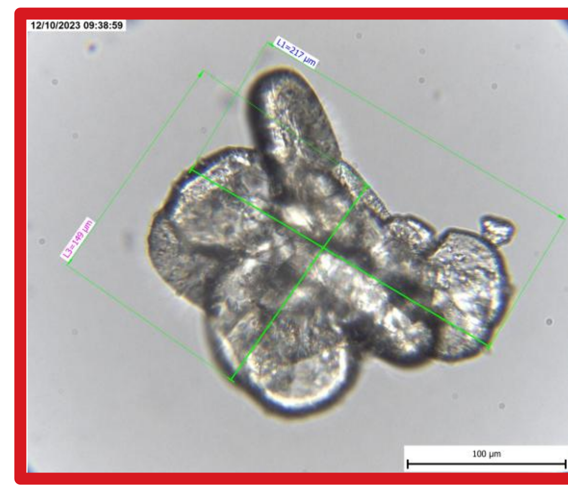


How Can We Spot LLPS?

- There are other **factors that indicate oiling**:
  - Consistent high concentration of sampled aliquots compared to the theoretical concentration.
  - Cloud points coinciding with clear points on phase diagrams.
  - Boulder-like solid.
  - Crowning and deposition on reactor vessel walls.
  - Fused crystals.

Isolation of boulder/rock like solids

Crowning and deposition on reactor wall or propeller



Fused crystals

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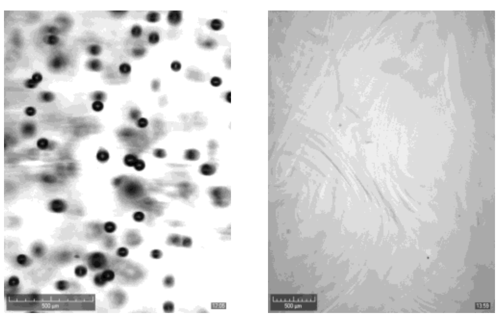
Why Does LLPS Occur?

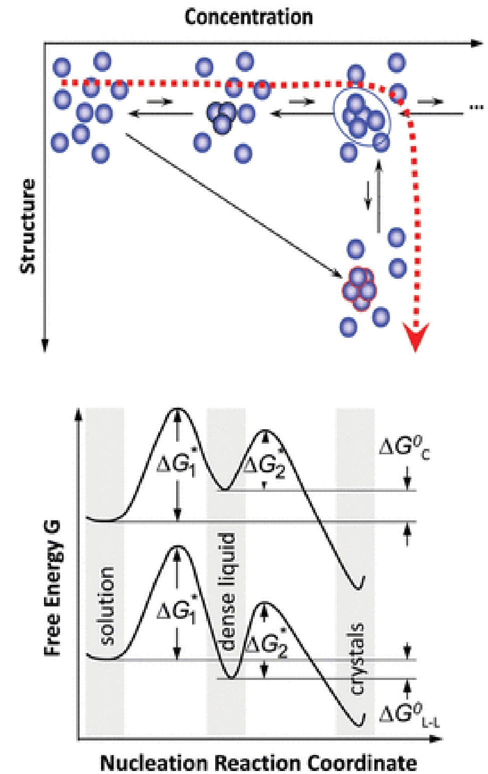
Gibbs Free Energy of Mixing

$\frac{\partial^2(\Delta G^{mix})}{\partial \chi^2} < 0 \rightarrow$ Spinodal decomposition mechanism.

$\frac{\partial^2(\Delta G^{mix})}{\partial \chi^2} > 0 \rightarrow$ Nucleation and growth mechanism.

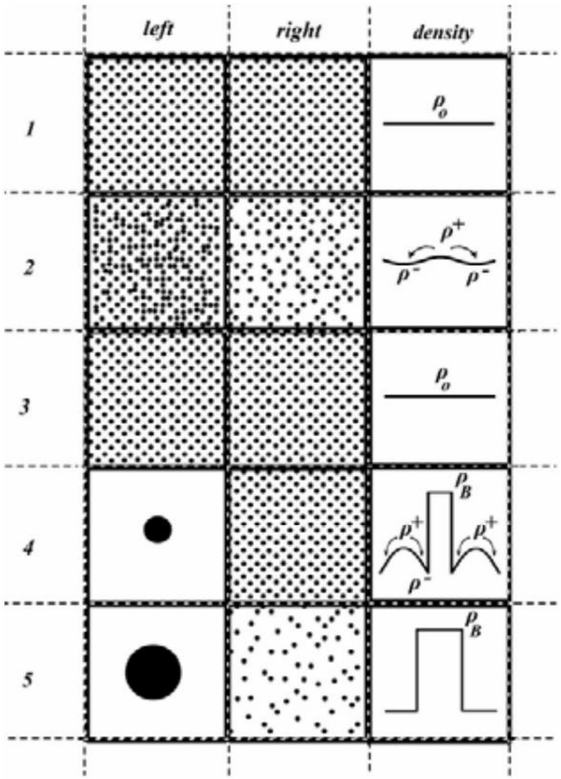


Two Step Nucleation Theory



Why Does LLPS Occur?
Nucleation and Growth Mechanism

- Gibbs Free Energy of Mixing describes two mechanisms of oil phase formation: **Spinodal decomposition** mechanism or **nucleation and growth** mechanism.
- Nucleation and Growth Mechanism:
  - The left and right columns represent two local regions in a solution of "B" brought to a metastable state.



1. No difference in the local concentration $C_B^{left} = C_B^{right}$ – no difference in density.
2. A very small fluctuation causes a concentration difference $C_B^{left} > C_B^{right}$.
3. Diffusion takes place from the higher to lower concentration (downhill) and the system is back to the initial stage.
4. A large fluctuation cause the formation of a nucleus of a **critical size**, resulting in a concentration of 0 in the left local region. $C_B^{left} = 0 < C_B^{right}$
5. Down-hill diffusion from right to left local regions takes place. Molecules reaching the left local region are captured by the nucleus and growth start taking place.

NOTE: Concentration refers to species concentrations not solute concertation

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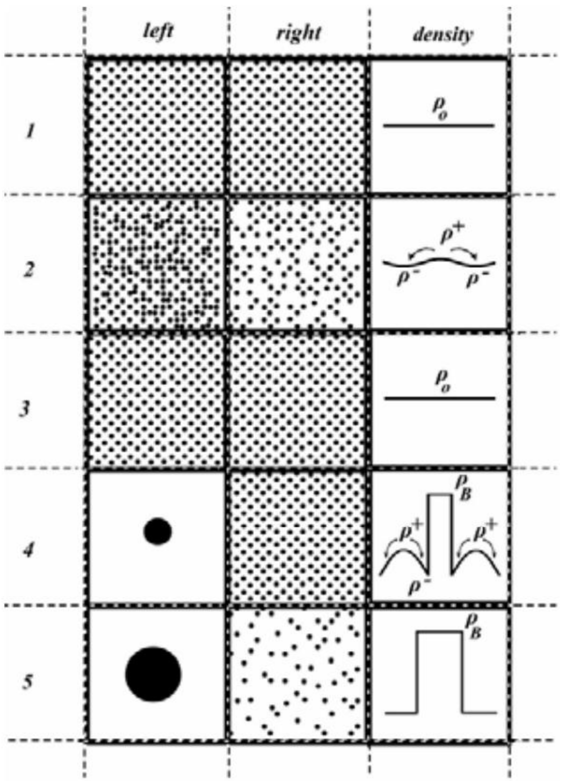
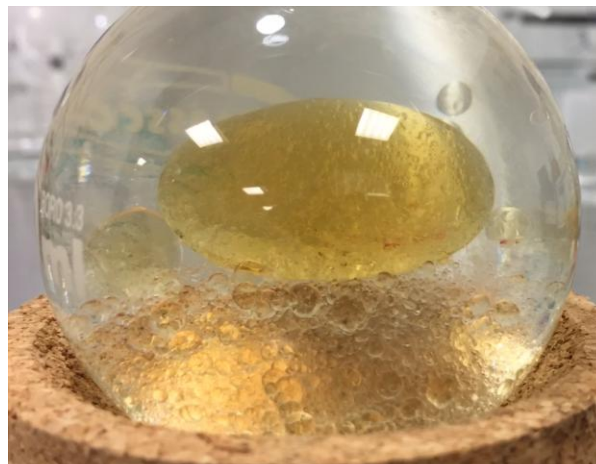
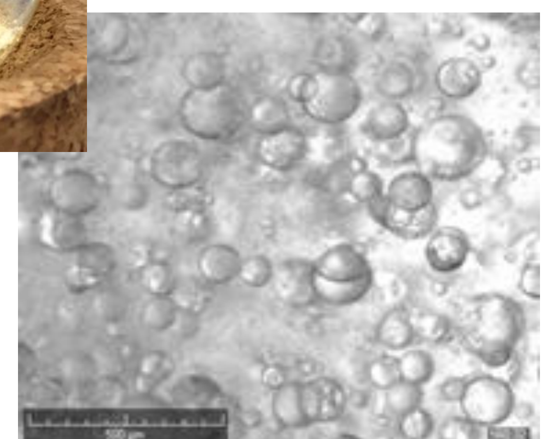
Why Does LLPS Occur? Nucleation and Growth Mechanism

- Gibbs Free Energy of Mixing describes two mechanisms of oil phase formation: **Spinodal decomposition** mechanism or **nucleation and growth** mechanism.
- Nucleation and Growth Mechanism:
  - This mechanism results in oil droplets.







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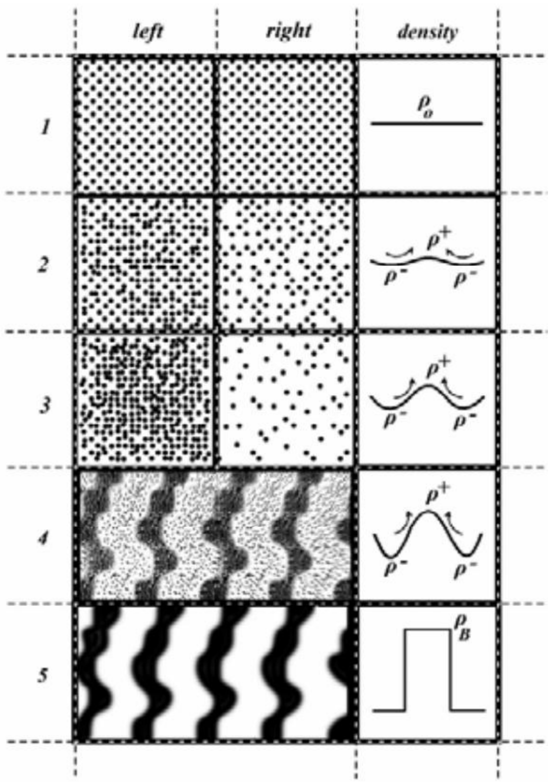
Why Does LLPS Occur?
Spinodal Decomposition Mechanism

- Gibbs Free Energy of Mixing describes two mechanisms of oil phase formation: **Spinodal decomposition** mechanism or **nucleation and growth** mechanism.
- Spinodal Decomposition Mechanism:
  - The left and right columns represent two local regions in a solution of “B” brought to a metastable state.

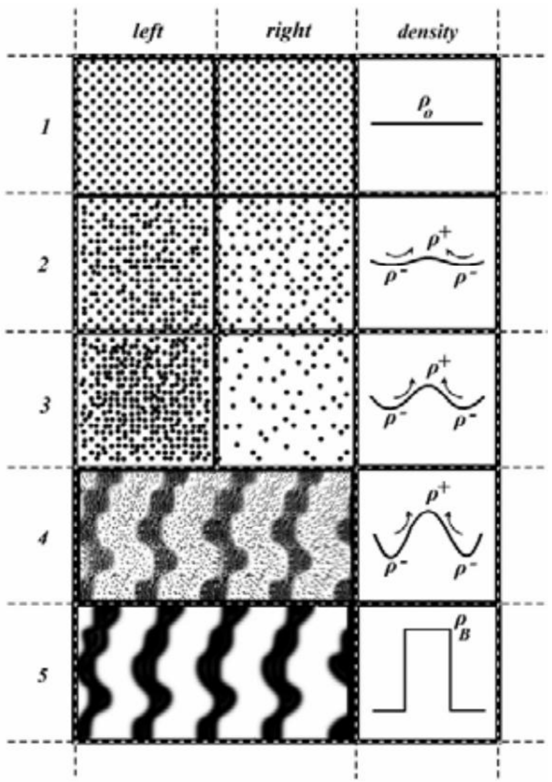


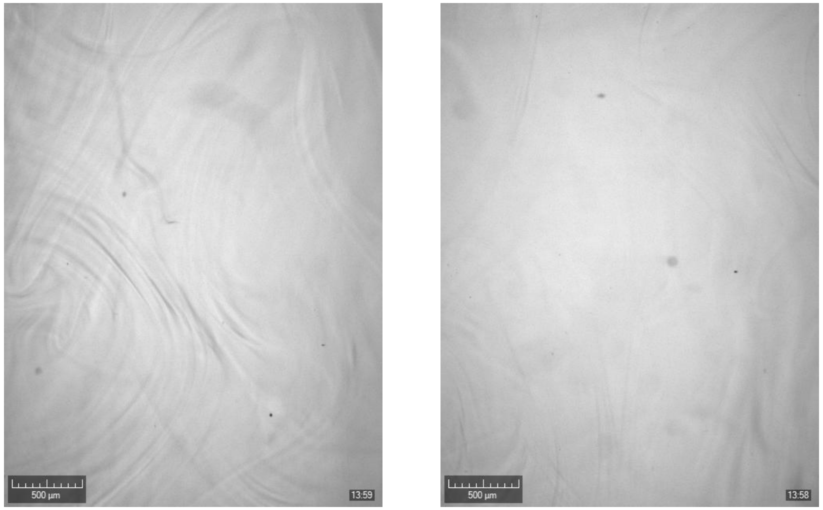
1. No difference in the local concentration $C_B^{left} = C_B^{right}$ – no difference in density.
2. A very small fluctuation causes a concentration difference $C_B^{left} > C_B^{right}$.
3. Diffusion does not take place as expected and the system showed an uphill diffusion profile resulting in $C_B^{left} >> C_B^{right}$.
4. Row 4 is produced by repeating row 3.
5. Row 5 is the end step of LLPS.

NOTE: Concentration refers to species concentrations not solute concertation

Why Does LLPS Occur?
Spinodal Decomposition Mechanism

- Gibbs Free Energy of Mixing describes two mechanisms of oil phase formation: **Spinodal decomposition** mechanism or **nucleation and growth** mechanism.
- Spinodal Decomposition Mechanism:
  - The defining characteristic of such mechanism is the uphill diffusion – making layers.

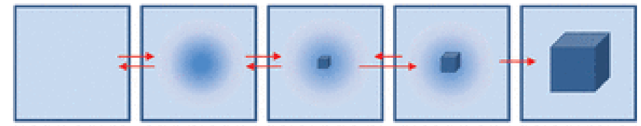




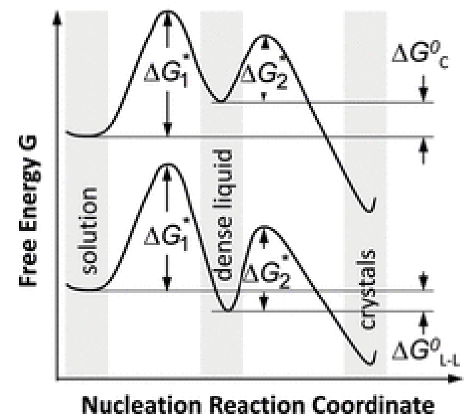
NOTE: Concentration refers to species concentrations not solute concertation

Why Does LLPS Occur?
Two Step Nucleation Theory

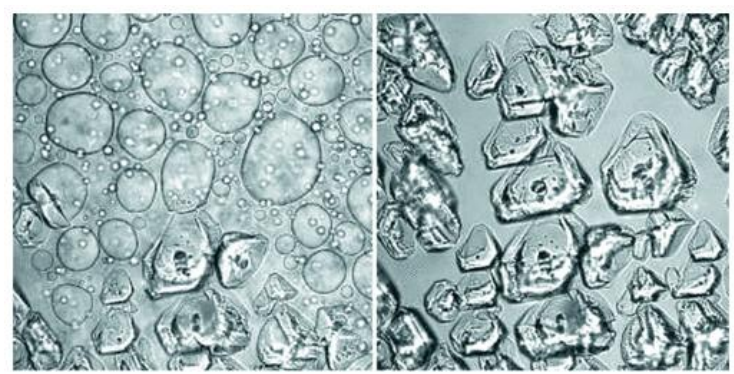
- **Two step nucleation theory** looks at the oil phase as a transitional state towards crystals.



1. Formation of an initial cluster of solutes that are rather loose in structure, often referred to as “**Dense Liquid Phase**”.



2. Reorganisation of molecules in the clusters into crystalline nuclei.
   – Re-organization of molecules may be slow due to high viscosity and low molecular mobility; seeding might help circumventing this.



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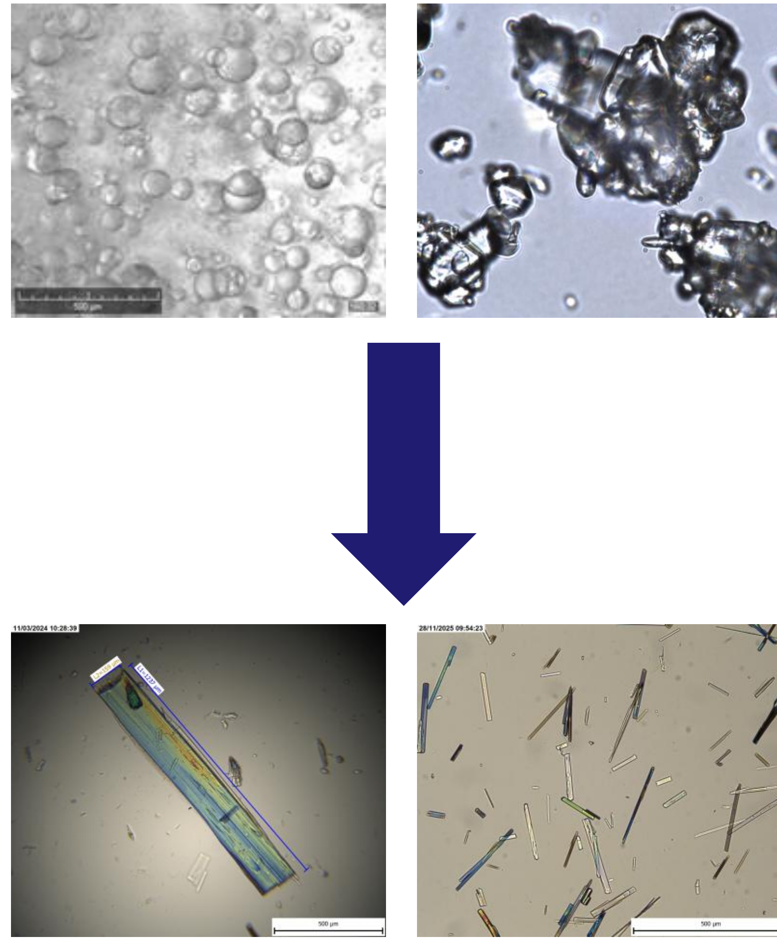
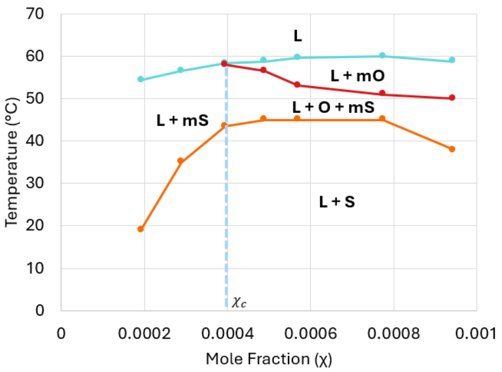


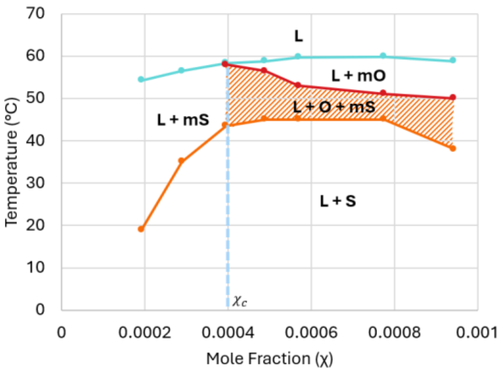
How Do We Avoid LLPS?

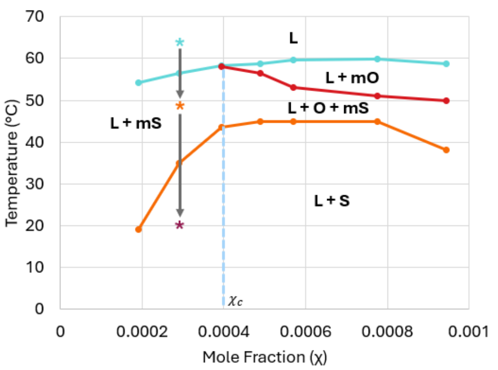
1. Plot a LLPS diagram

2. Identify any oiling regions

3. Plan a crystallisation pathway which avoids the oiling region







How Do We Avoid LLPS?

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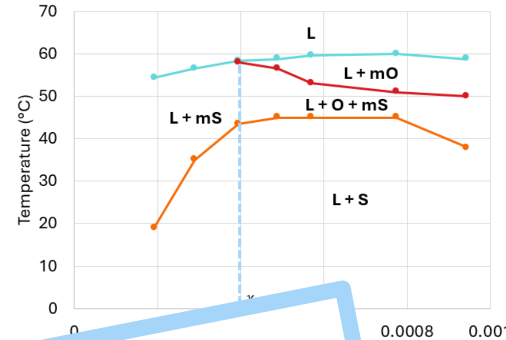
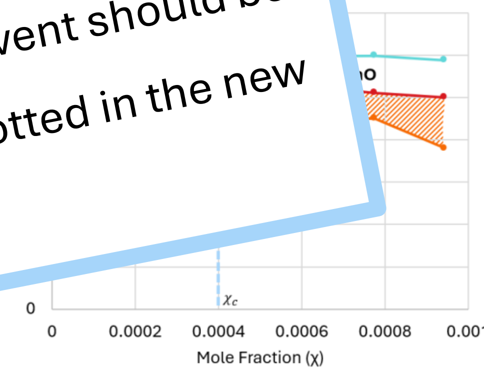
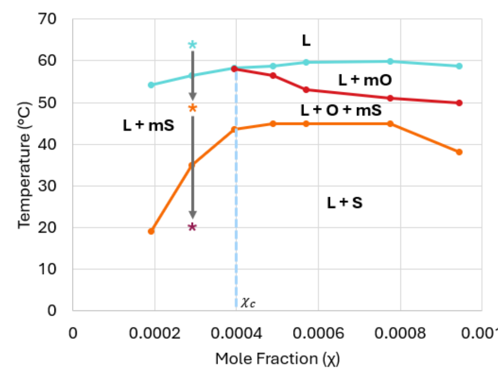


2

If no safe region is found, the solvent should be swapped and LLPS diagrams plotted in the new solvent system



3. Plan a crystallisation pathway which avoids the oiling region

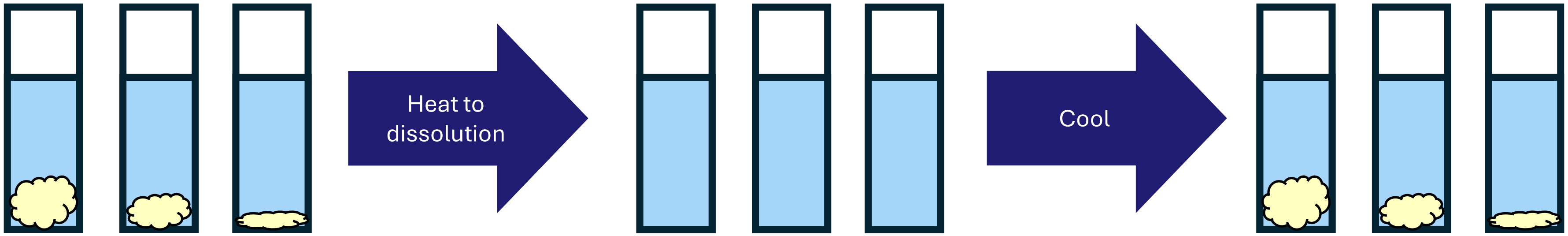


How Do We Plot LLPS Diagrams?

- The **Technobis Crystalline** is used to plot LLPS Diagrams
- It allows 8 vials to be individually agitated and temperature controlled
- It has in-situ cameras which take images of the vials throughout an experiment to provide information about clear and cloud points
- It has lasers to monitor transmissivity throughout an experiment

How Do We Plot LLPS Diagrams?

1. Prepare suspensions of different concentrations in a chosen solvent system (concentrations which you are confident will fully dissolve on heating to the chosen high temperature).
2. Use the **Crystalline** to slowly heat the vials.
3. Slowly cool the vials to room temperature.
4. Images are taken of the vials frequently throughout experiments.



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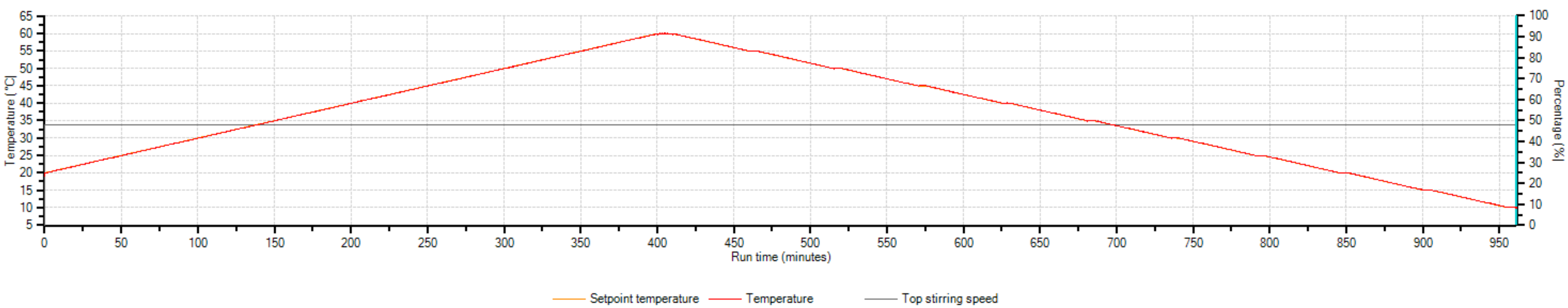

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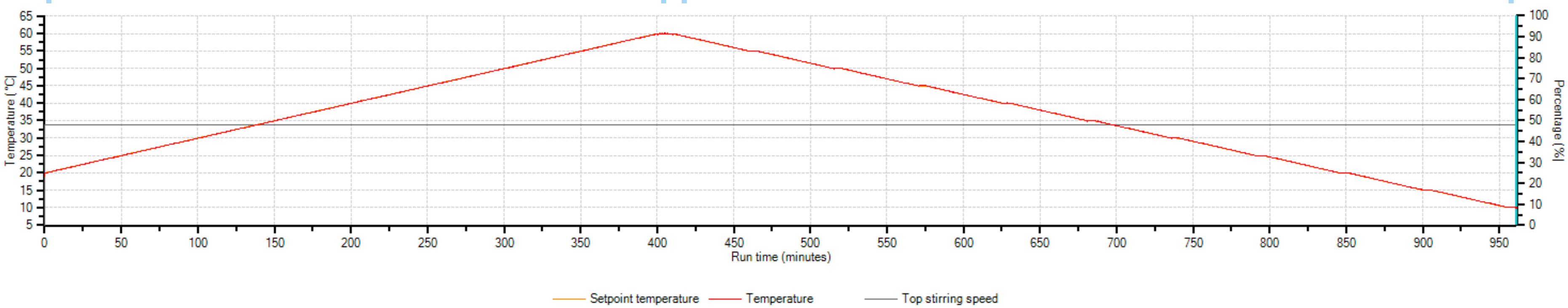
3. Slowly cool the vials to room temperature.
4. Images are taken of the vials frequently throughout experiments.

Clear points are identified through the heating step

Cloud points are identified through the cooling step



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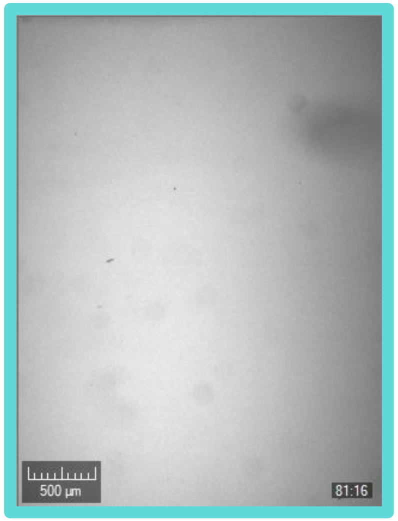
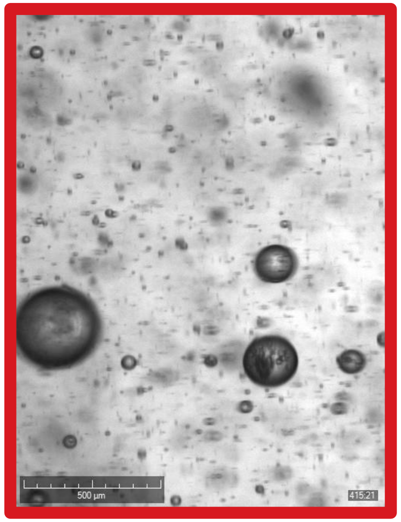
How Do We Plot LLPS Diagrams?

- Look through the in-situ **Crystalline** images to identify clear and cloud points for each concentration.
  - Clear points - temperatures at which all solid had dissolved, the solution becomes a single-phase liquid.
  - Cloud points due to LLPS – temperature at which oil is first observed.
  - Cloud points due to crystallisation – temperature at which solid first nucleated.



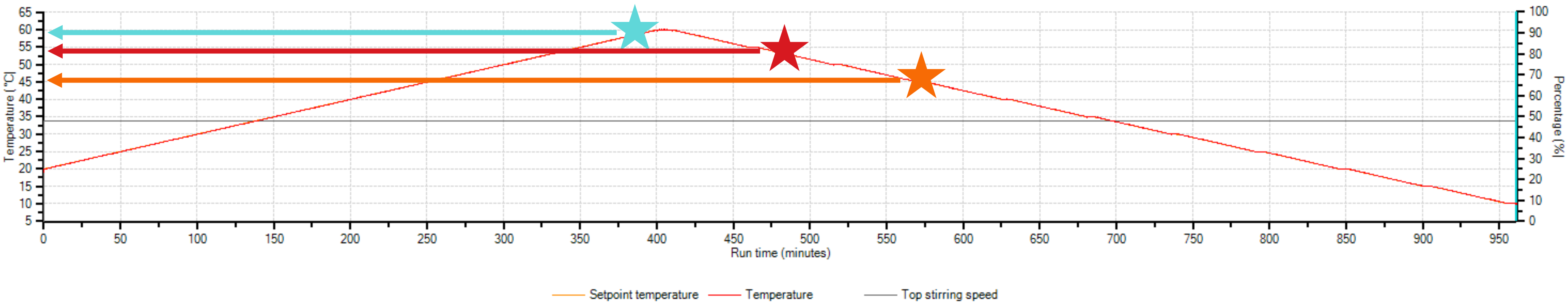
Liquid

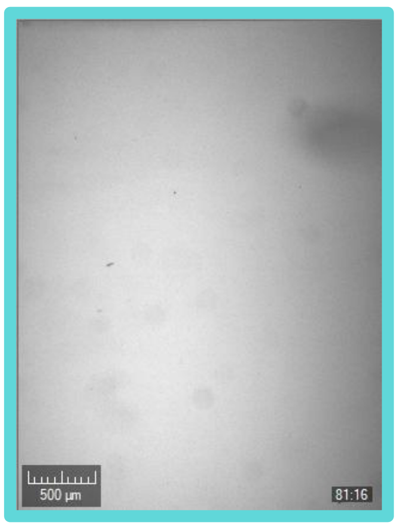


Oil

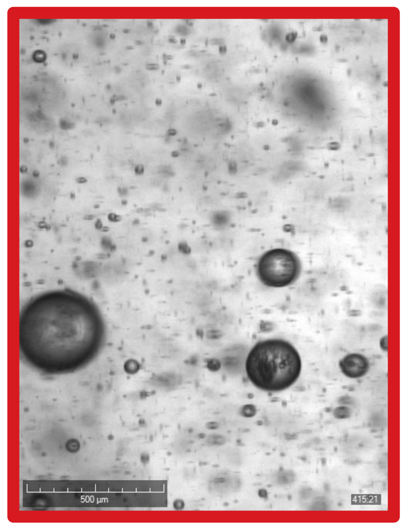
Solid

How Do We Plot LLPS Diagrams?

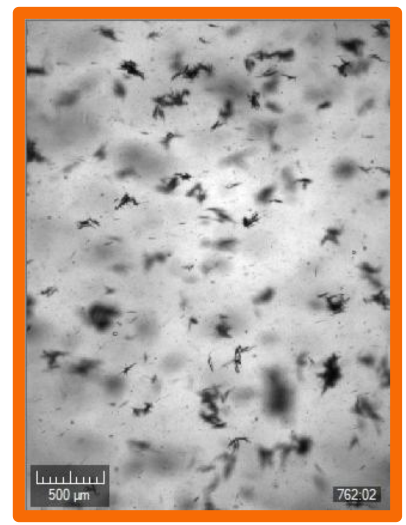




59.7 °C



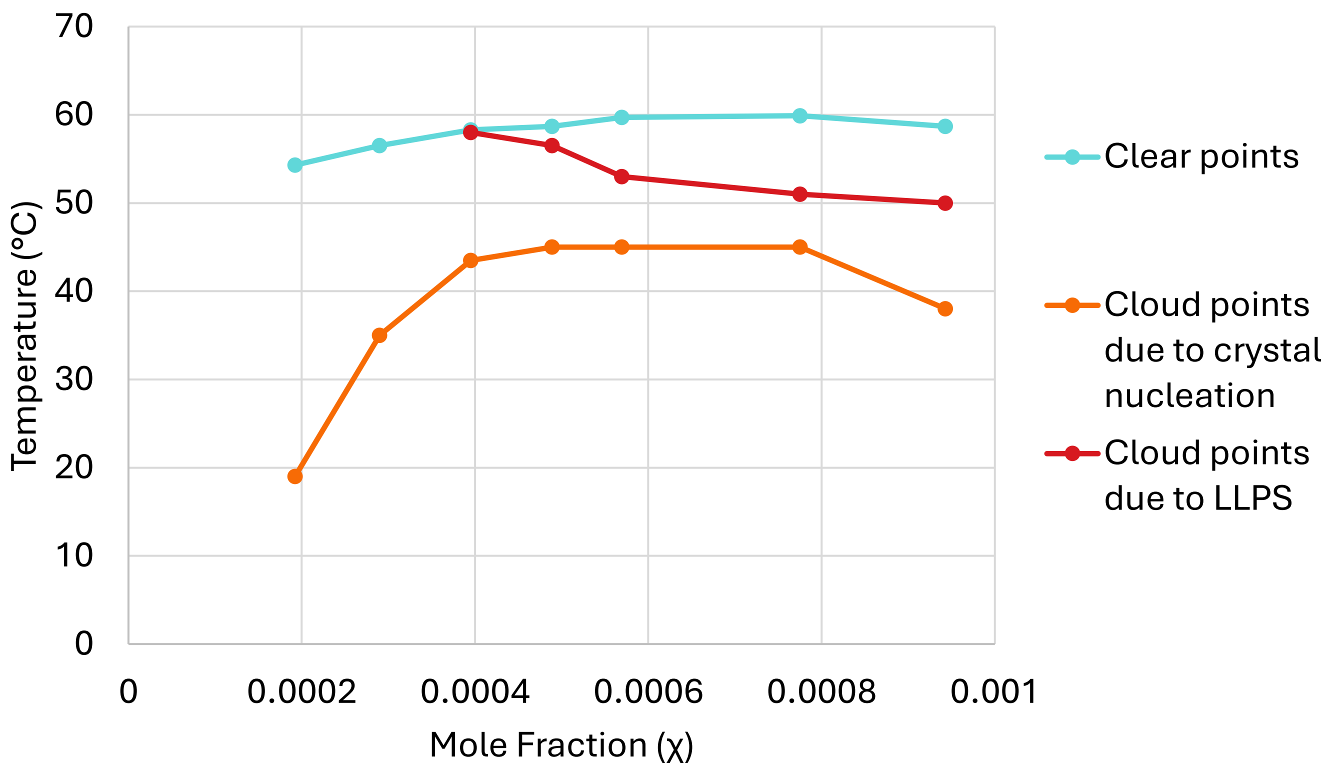
53.0 °C



45.0 °C

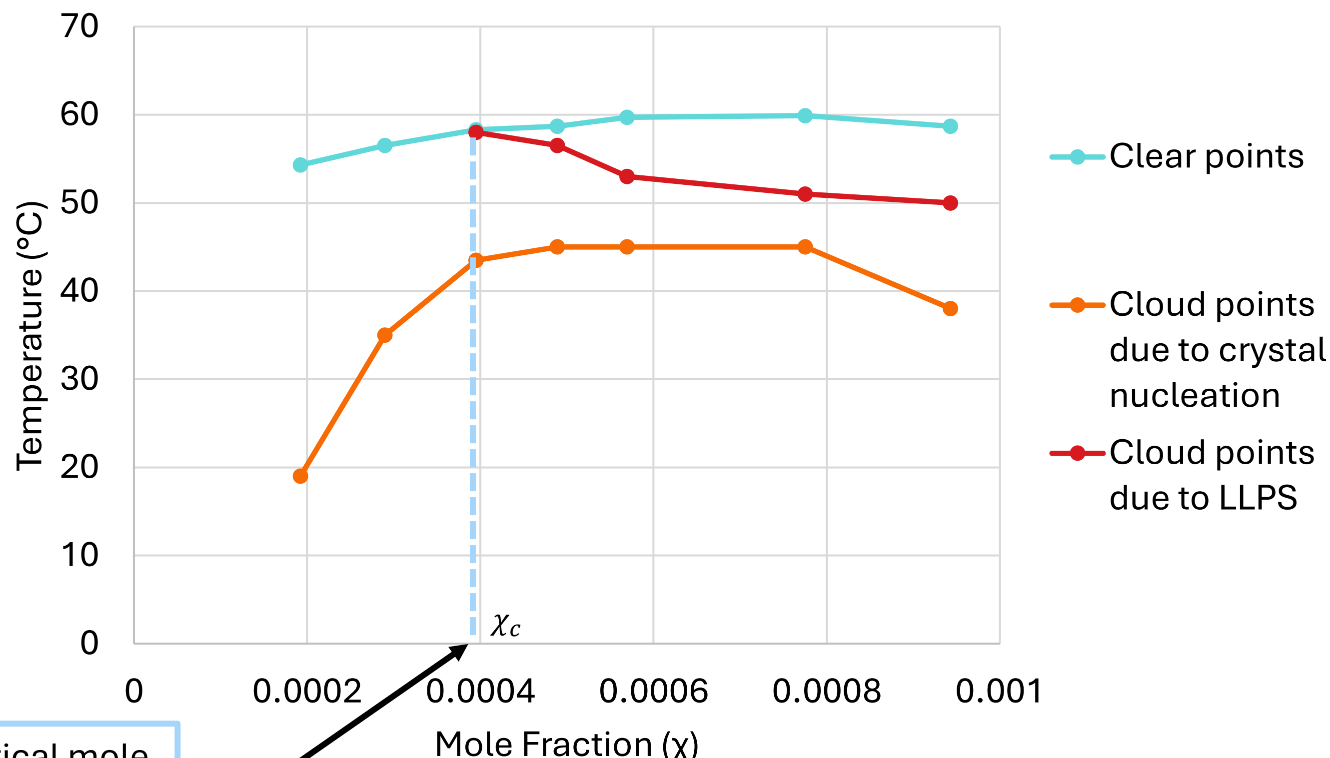
How Do We Plot LLPS Diagrams?

- Once clear and cloud points have been identified for each concentration, they can be plotted on a graph.
- They can either be plotted against mole fraction or concentration, depending on what is of interest.



How Do We Plot LLPS Diagrams?

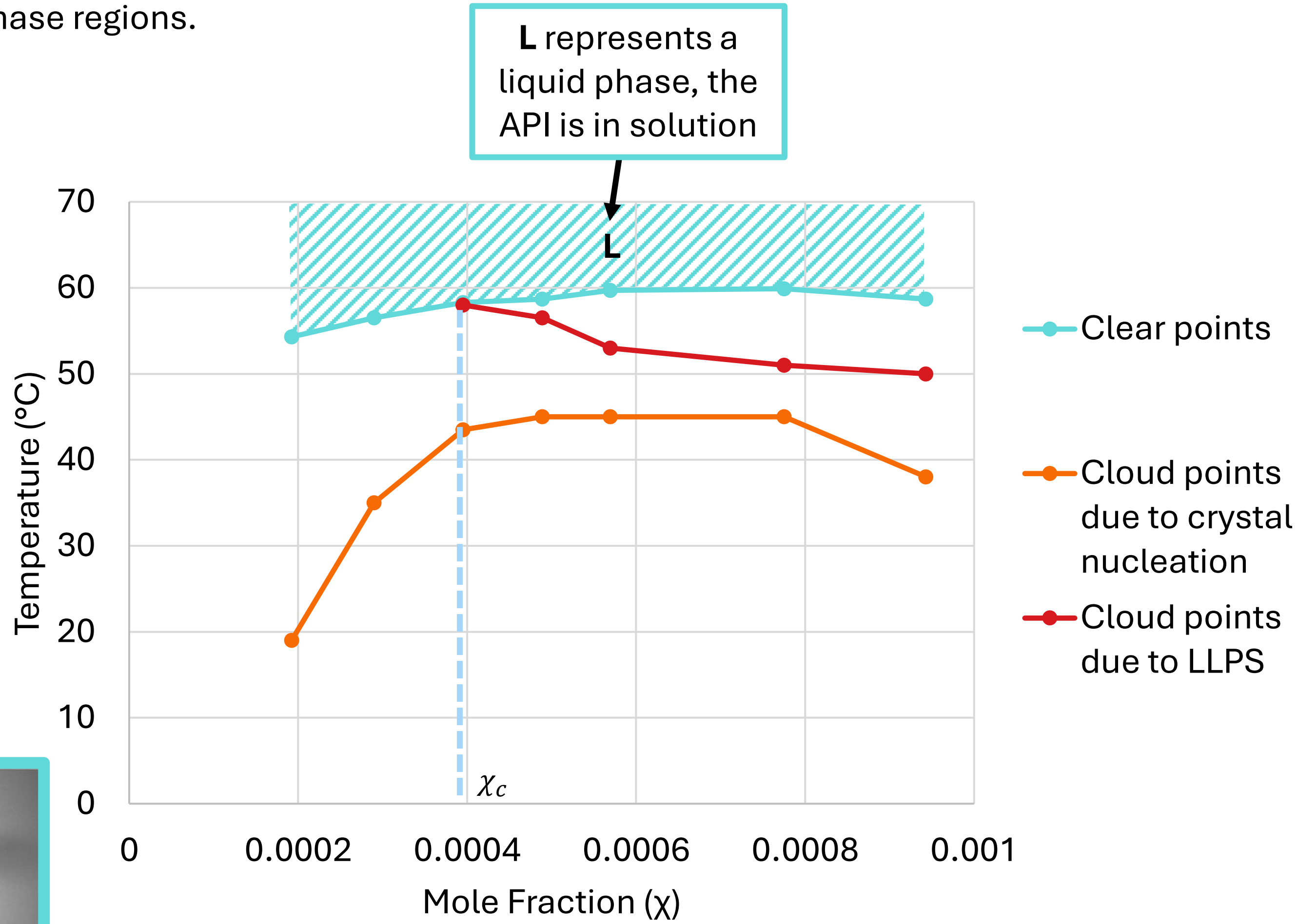
1. Identify any critical mole fractions (χ) – mole fractions where clear and cloud points overlap.

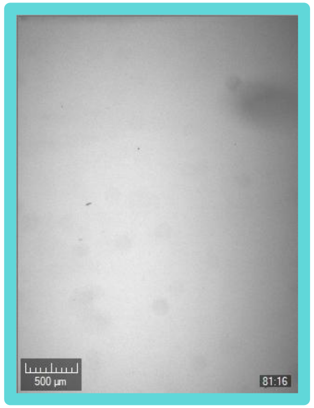


χ_c is the critical mole fraction, the mole fraction at which clear points and cloud points overlap

How Do We Plot LLPS Diagrams?

3. Identify phase regions.

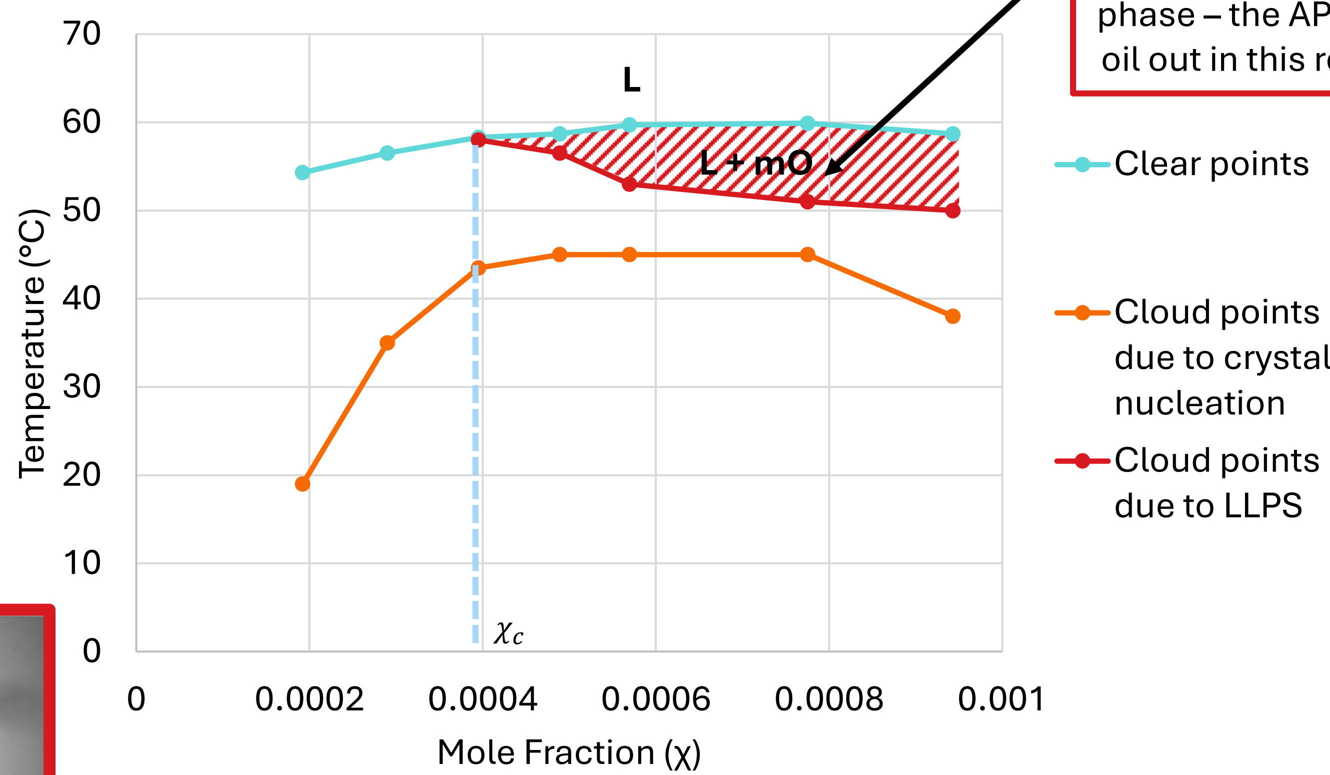


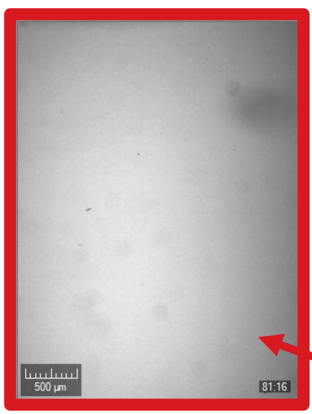


How Do We Plot LLPS Diagrams?

3. Identify phase regions.

L+mO represents an equilibrium between a liquid and metastable oil phase – the API may oil out in this region



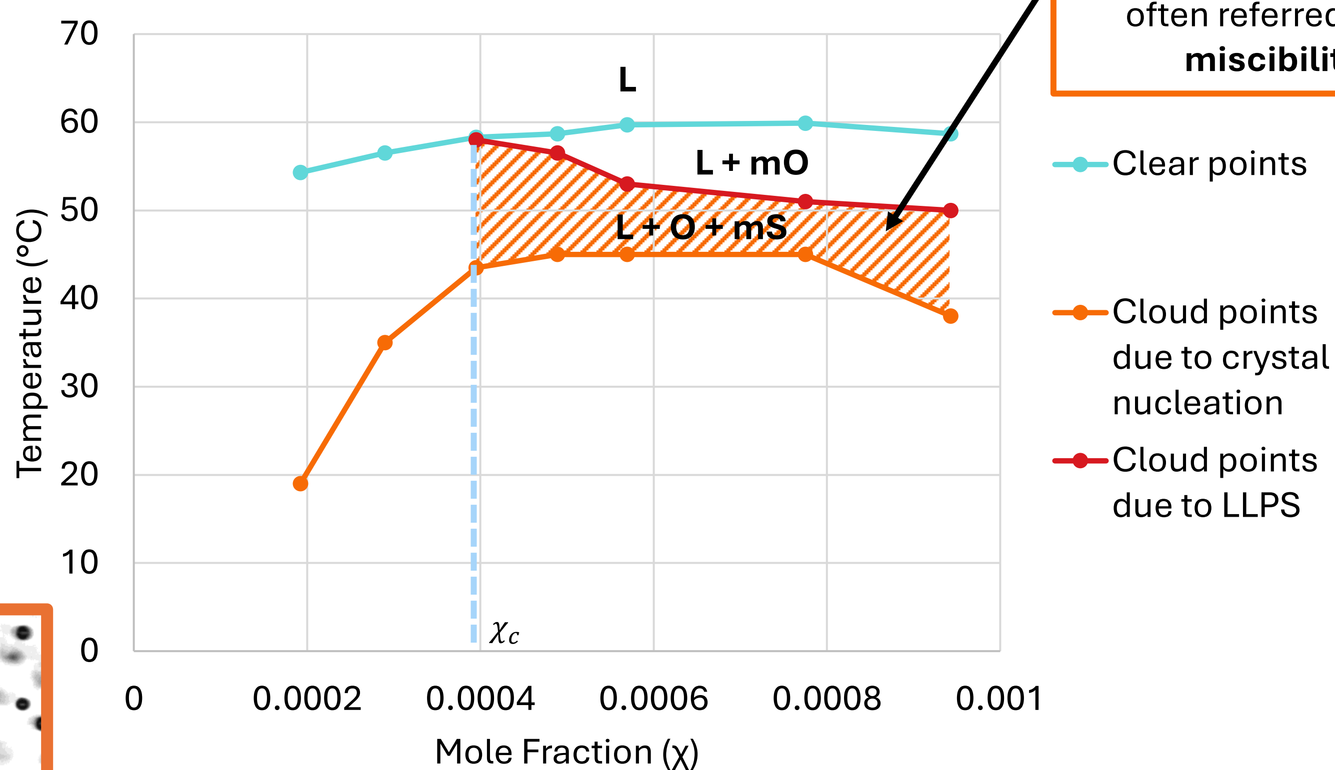


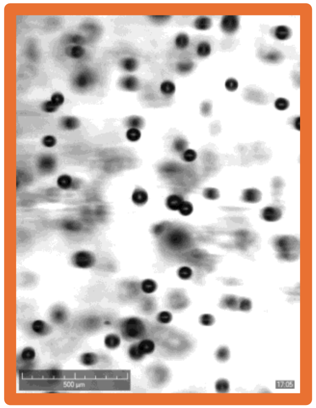
Will still appear liquid in this region unless seeded as the oil is metastable

How Do We Plot LLPS Diagrams?

3. Identify phase regions.

L+O+mS represents an equilibrium between a liquid, oil and metastable solid phase. The oiling region is often referred to as the **miscibility gap**

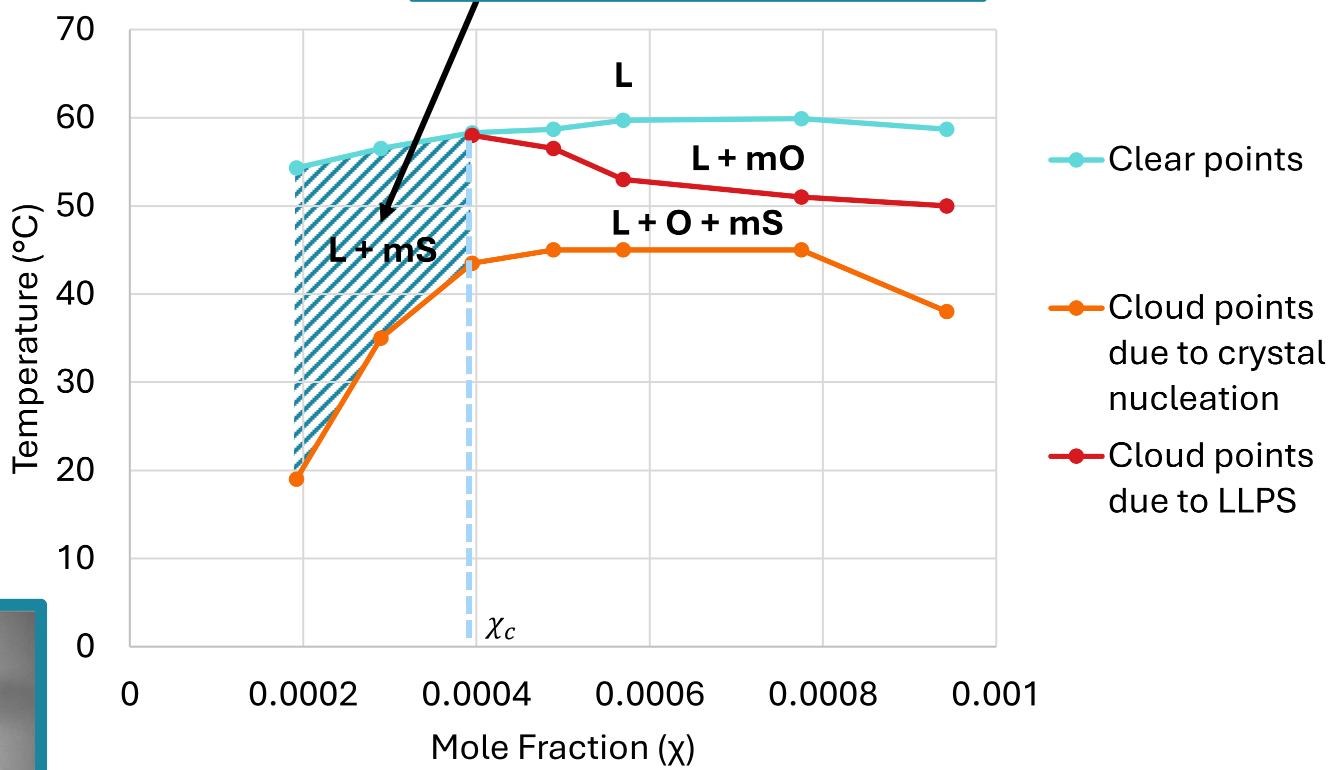


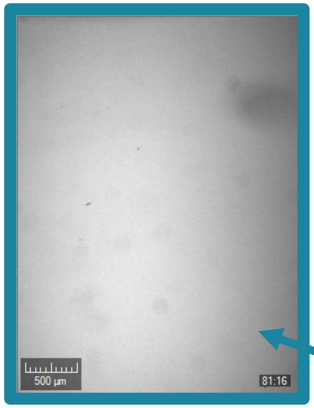


How Do We Plot LLPS Diagrams?

3. Identify phase regions.

L+mS represents an equilibrium between a liquid and metastable solid phase – usually referred to as the **metastable zone width**

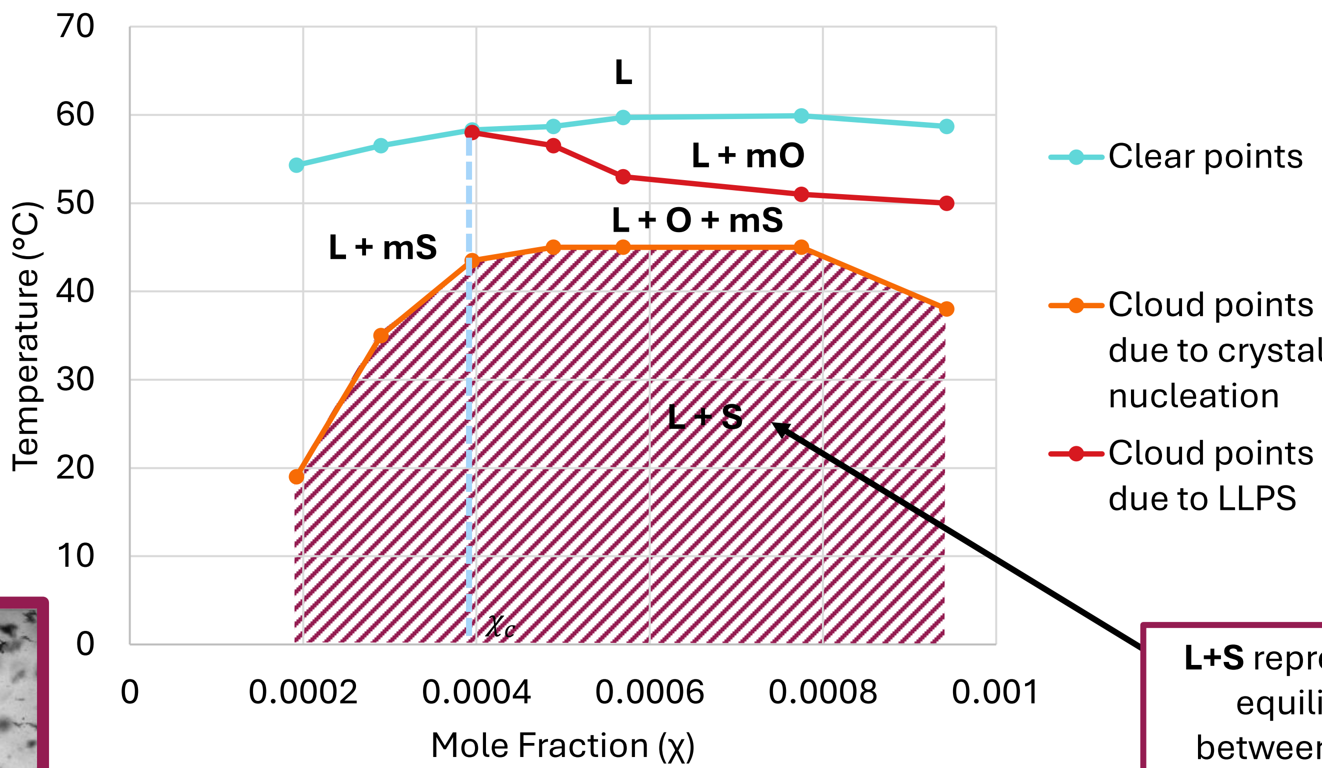




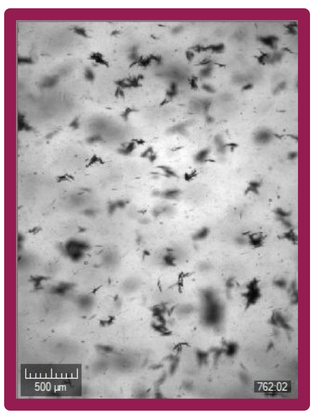
Will still appear liquid in this region unless seeded as the solid is metastable

How Do We Plot LLPS Diagrams?

3. Identify phase regions.

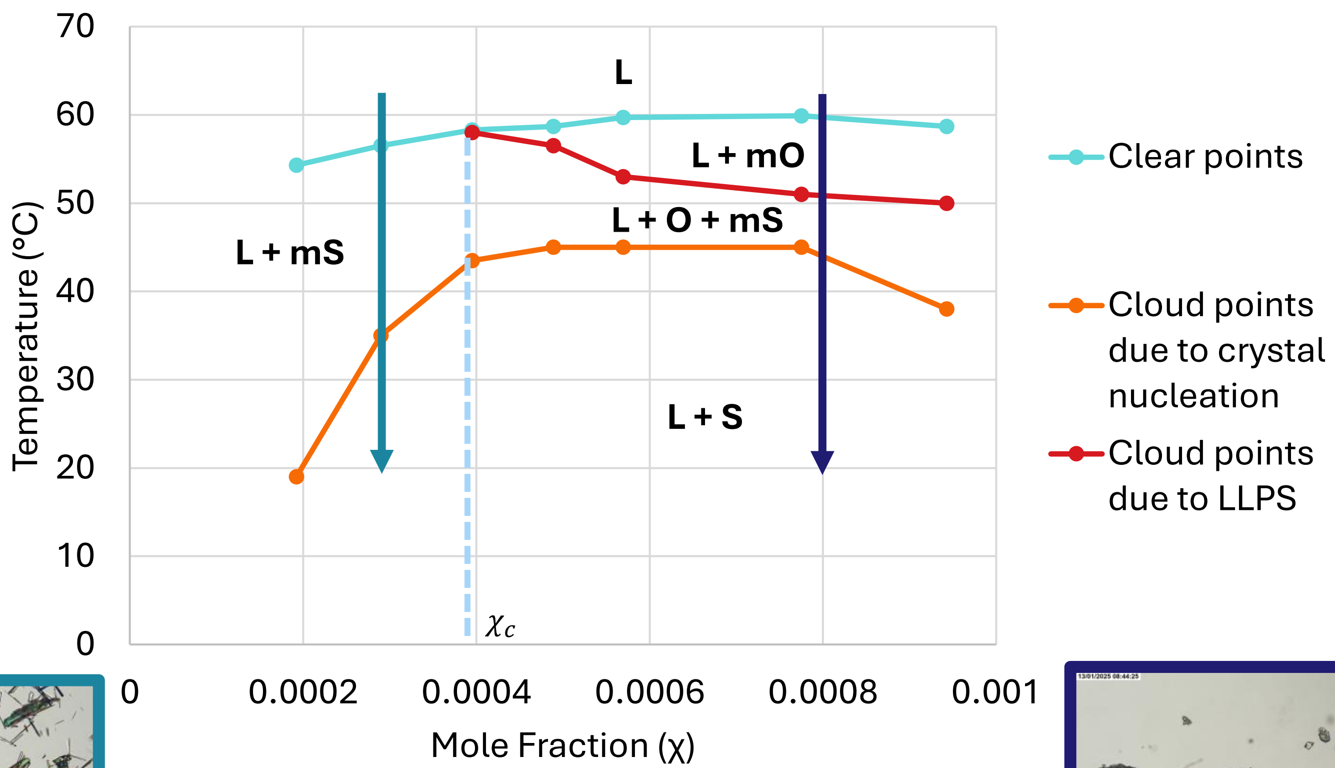


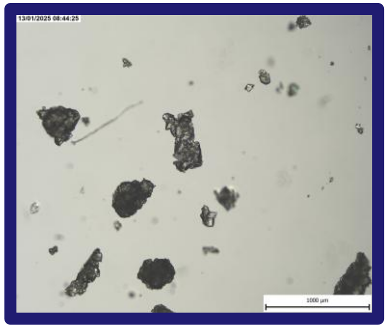
L+S represents an equilibrium between a liquid and a solid phase - a slurry



How Do We Plot LLPS Diagrams?

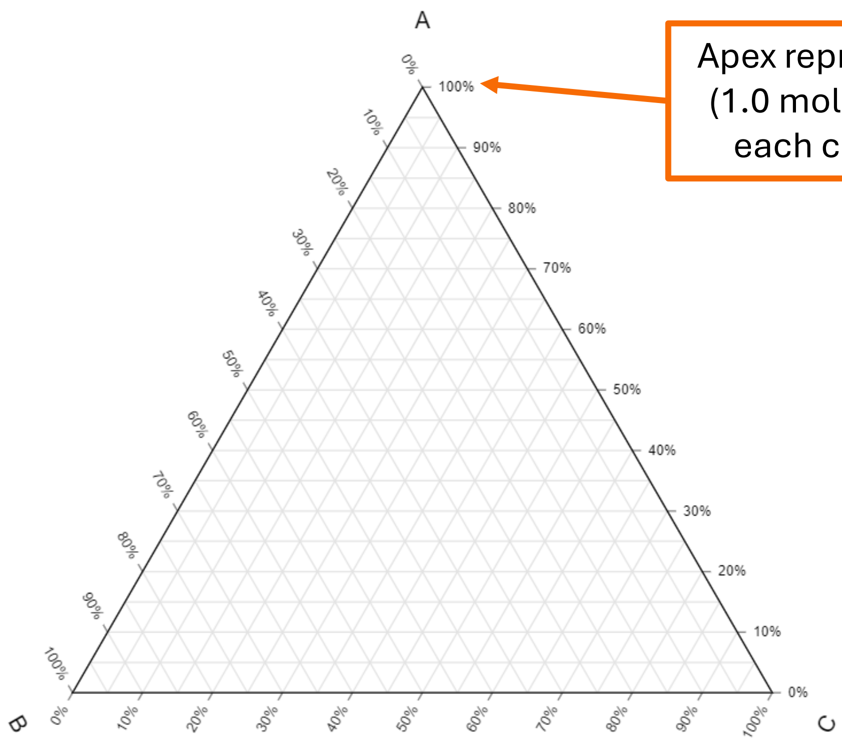
- Traversing through the **L+mS** phase, avoiding the oil, will result in single crystals.
- Going through the oil phase will likely result in aggregated crystals/clusters.





Triangular Phase Diagrams

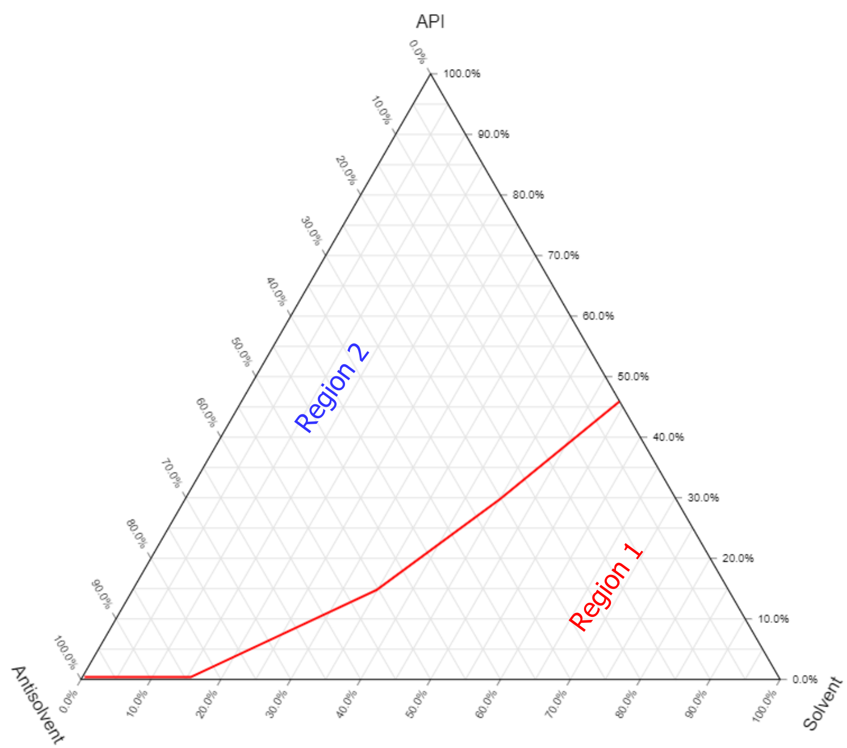
- LLPS often takes place in solvent:anti-solvent systems.
- To present the phase behaviour of solutions with different solvent ratios, triangular phase diagrams are plotted.



Apex represents 100% (1.0 mole fraction) of each component.

Triangular Phase Diagrams

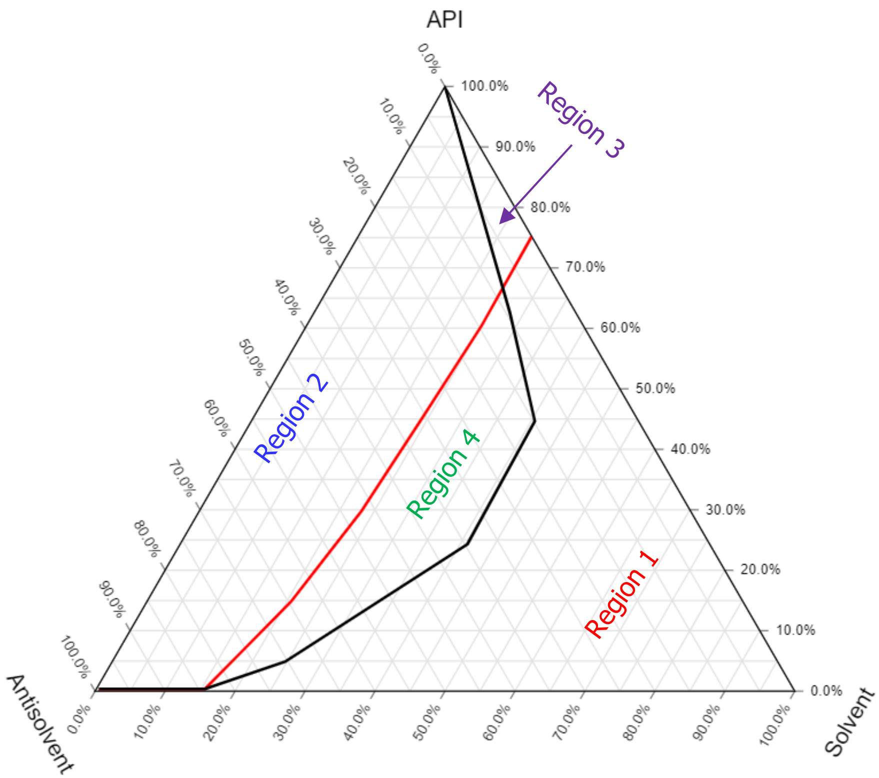
- Measuring the solubility of a molecule in different compositions at **fixed temperature**, we can plot the phase diagram below consisting of two regions.
- Region 1: liquid phase – API is in solution.
- Region 2: solid-liquid equilibrium (SLE-1) – slurry (nucleation zone).



5 °C

Triangular Phase Diagrams

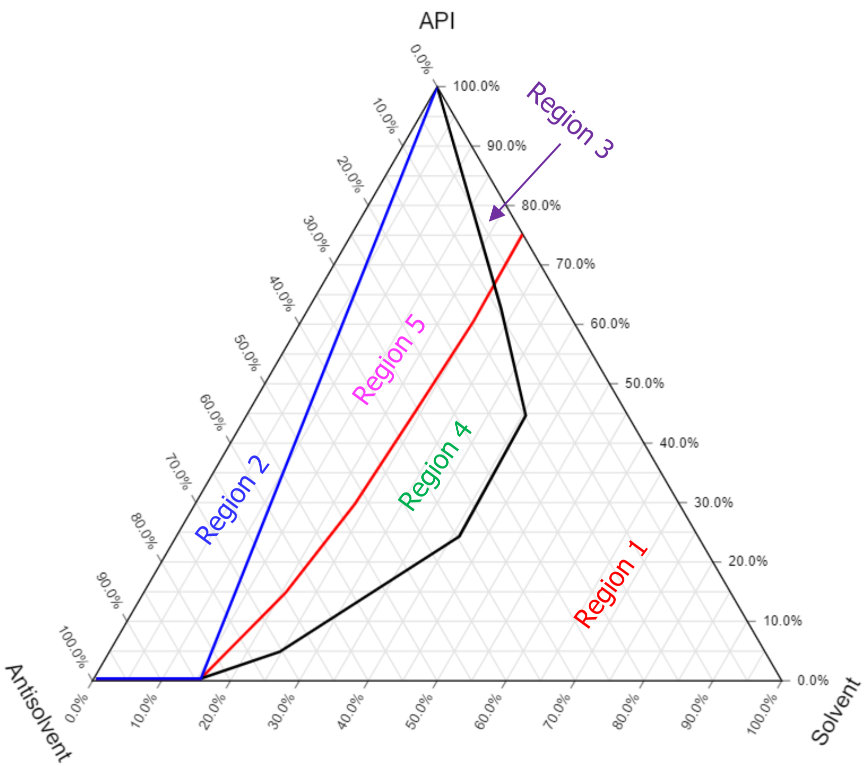
- Measuring of solubility in different compositions at **higher temperature**, can result in the phase diagram below consisting of multiple regions.
- Region 1: liquid phase – API is fully soluble.
- Region 2: solid-liquid equilibrium (SLE-1) – slurry.
- Region 3: solid-liquid equilibrium (SLE-2) – slurry.
- Region 4: liquid-liquid equilibrium (LLE) – LLPS region as an oil phase and a solvent phase.



20 °C

Triangular Phase Diagrams

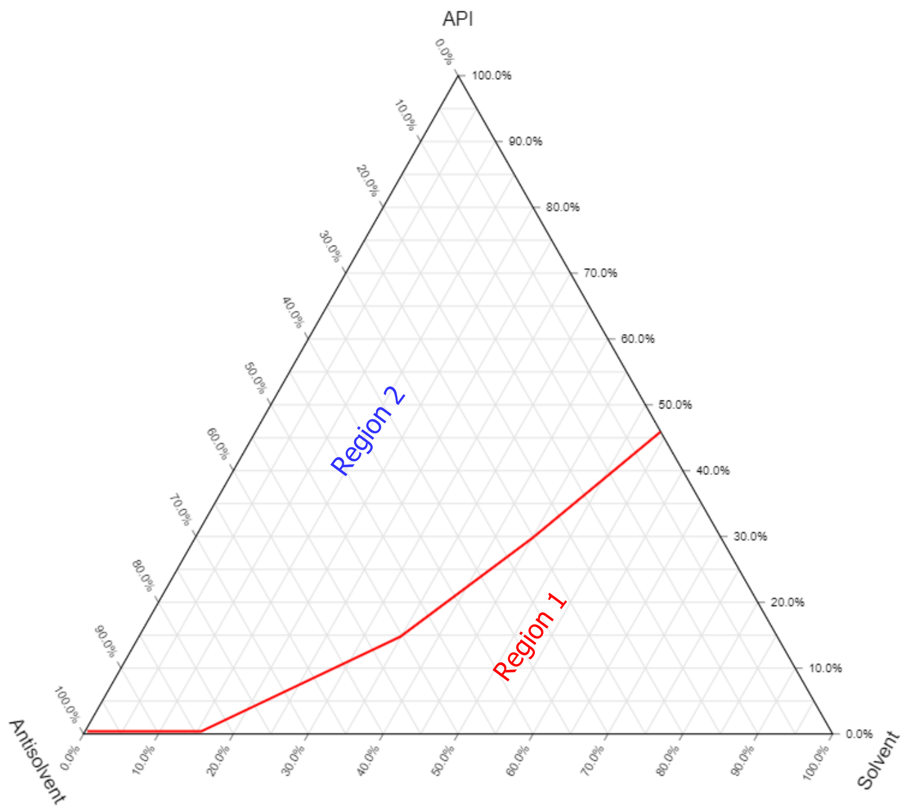
- Region 1: liquid phase – API is fully soluble.
- Region 2: solid-liquid equilibrium (SLE-1) – slurry.
- Region 3: solid-liquid equilibrium (SLE-2) – slurry.
- Region 4: liquid-liquid equilibrium (LLE) – LLPS region as an oil phase and a solvent phase.
- Region 5: solid-liquid-liquid equilibrium (SLLE) – one solid phase and two liquid phase coexist.



20 °C

Triangular Phase Diagrams

- In a system with two regions (no oiling region), cooling or adding antisolvent will result in crystallisation.



Region 1: liquid phase – API is fully soluble.

Region 2: solid-liquid equilibrium (SLE-1) – slurry.

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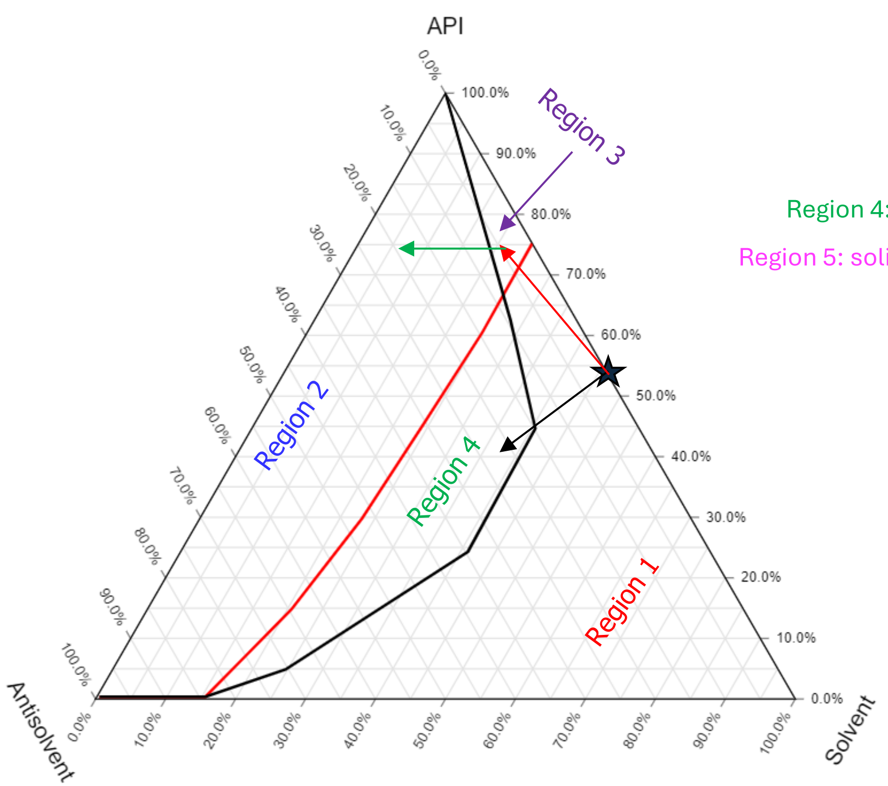
Triangular Phase Diagrams

- If addressing a system with regions 1, 2, 3 and 4, adding antisolvent will result in LLPS (region 4).
- Cooling the system first will move the composition into region 3 (SLE-2).
- From there adding antisolvent will move the system equilibrium into region 2.



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Region 4: liquid-liquid equilibrium (LLE) – LLPS region as an oil phase and a solvent phase.

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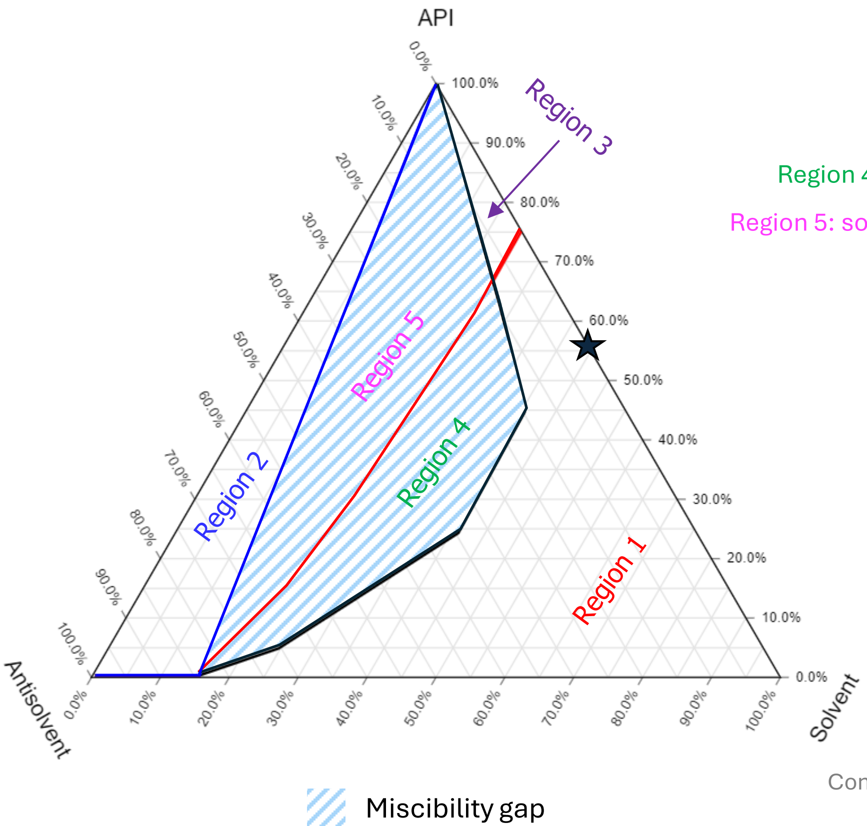
Triangular Phase Diagrams

- When we have all five regions, traversing from region 1 to region 2 without going through an oil phase is impossible.



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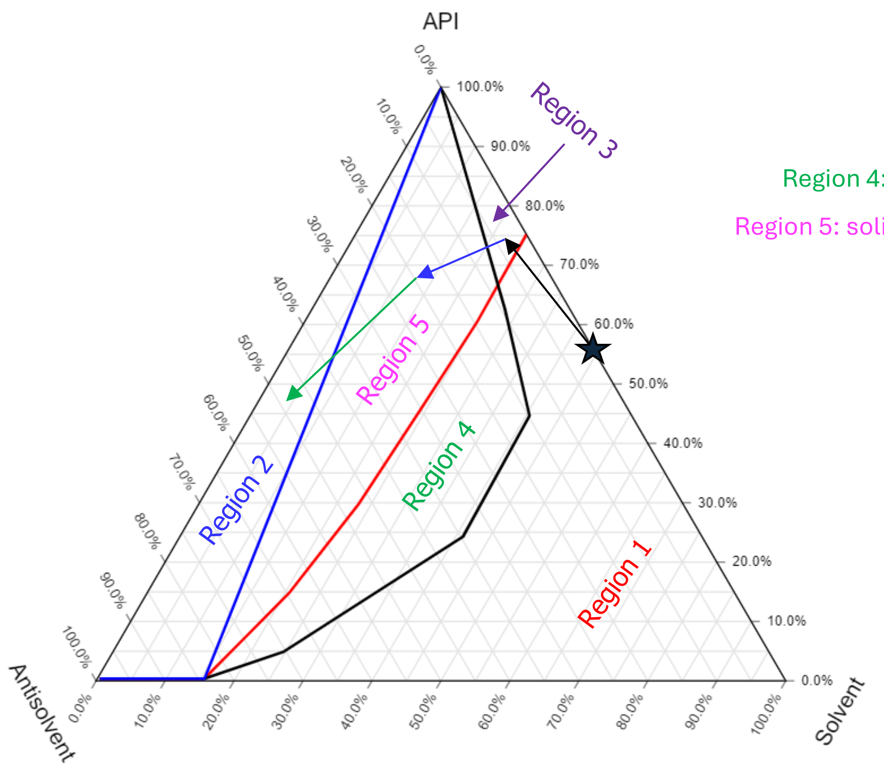
Triangular Phase Diagrams

- We must go through region 3 to avoid region 4 by cooling.
- Charging antisolvent will take us into region 5 (SLLE).
- Adding a sufficient hold in region 5 can help transforming the oil phase to solids before moving to region 2.



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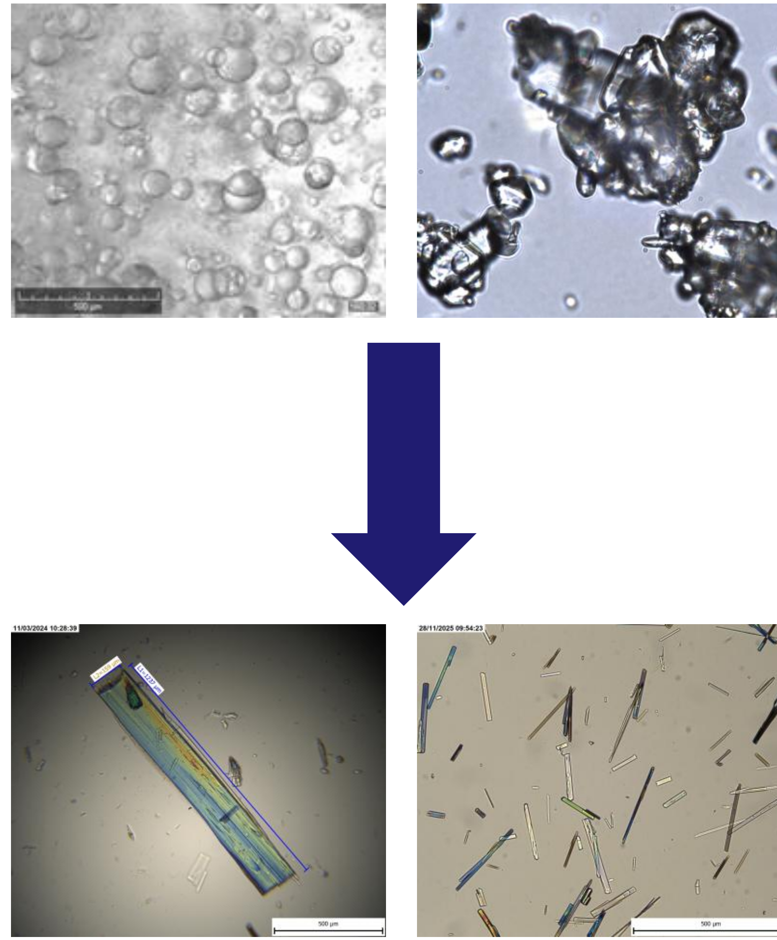
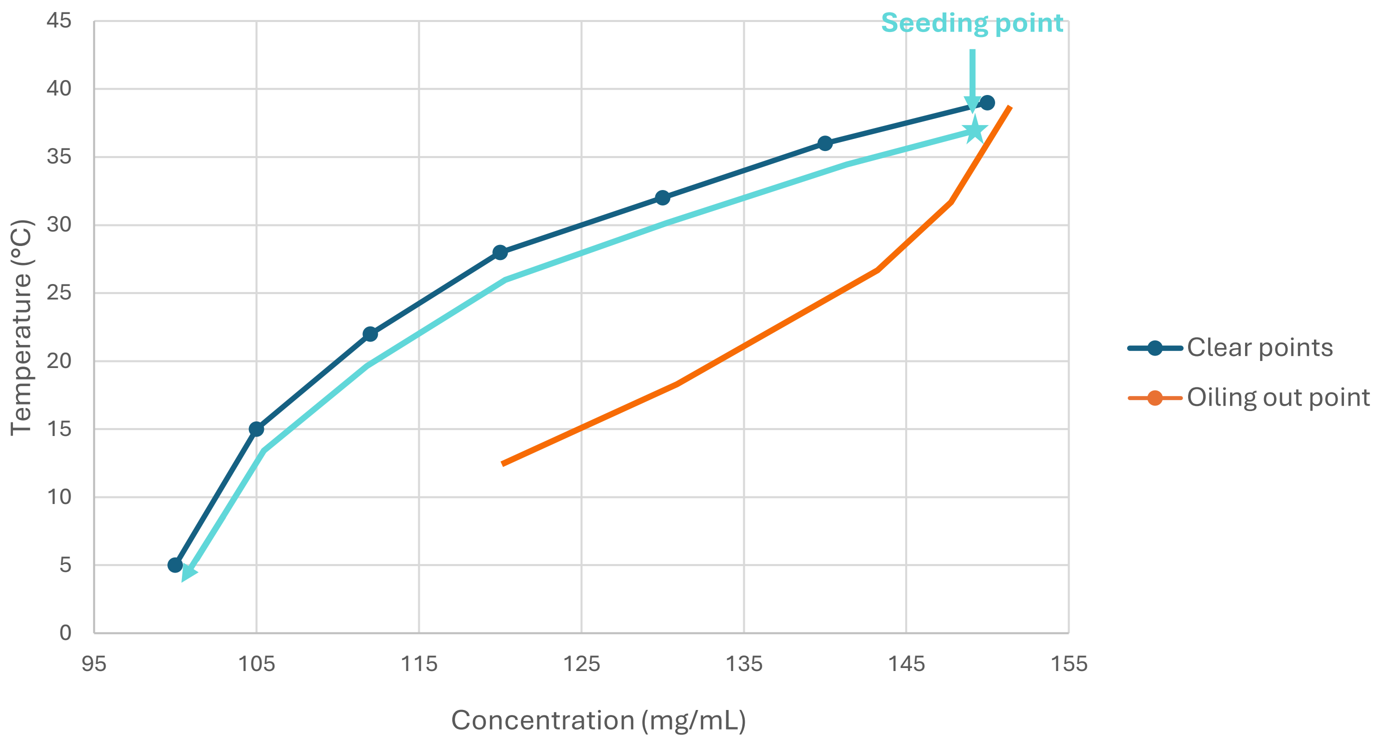
Contents



Avoiding Oiling - Seeding and Supersaturation Control

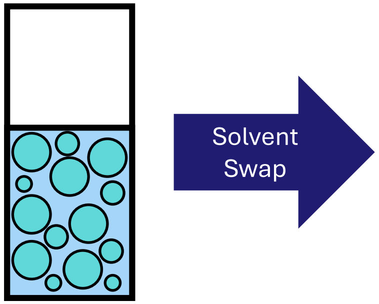
- Oiling can be mitigated by getting to a solid meta-stable zone of a supersaturated solution.
- Then crystal growth can be sustained in this region by adjusting the antisolvent composition, cooling rate and seed loading.
- This can occur by carefully maintaining the crystallisation procedure between the oiling out curve and the solubility curve.



Avoiding Oiling – Other Strategies

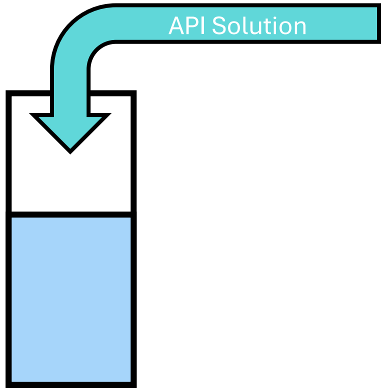
Solvent Switch

- According to regular solution model, solvent-solute interaction relative to solute-solute and solvent-solvent interactions plays a critical role in determining the mixing behaviour.
- Switching to different solvent is a common strategy to avoid LLPS.



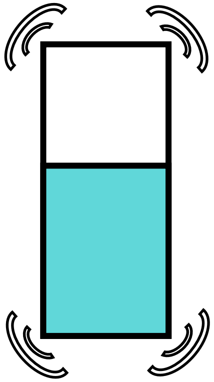
Reverse Addition

- Adding a solution of API to an antisolvent rich phase with seed can result in avoiding LLPS.
- This is obtained as the concentration of the solute is maintained low (reducing the supersaturation level).



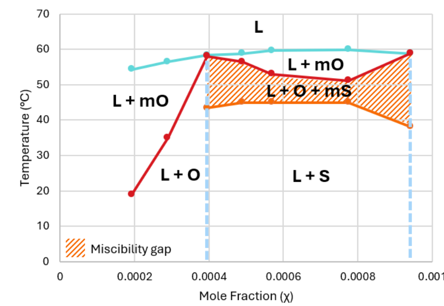
Ultrasound

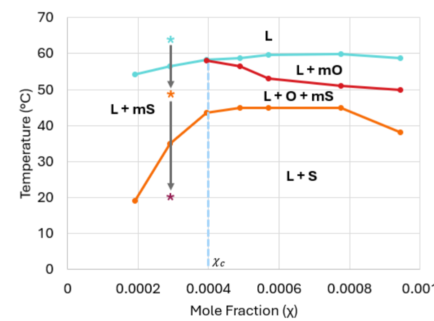
- Ultrasound can be used as an alternative to adding a seed.
- Ultrasound results in the creation of a small nuclei at relatively low supersaturation levels by insonation.

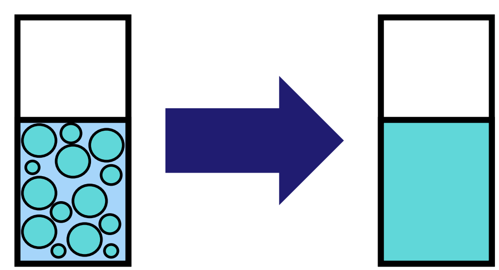


Conclusions

- LLPS (oiling out) is a key crystallisation risk that impacts particle quality and process robustness.
- The **Technobis Crystalline** is a crucial instrument in observing oiling and identifying conditions where it occurs.
- LLPS diagrams are essential tools to:
  - Identify oiling/miscibility gaps
  - Map phase behaviour
  - Design safe crystallisation routes
- Pathway matters:
  - Avoid oiling region → well-defined crystals
  - Through oiling region → aggregates / poor solids / deposition
- LLPS can be managed or mitigated via:
  - Process design (cooling, addition strategy)
  - Supersaturation control and seeding
  - Solvent selection or switching







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