Multiphase complex coacervate droplets

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Liquid-liquid phase separation plays an important role in cellular organization. Many subcellular condensed bodies are hierarchically organized into multiple coexisting domains or layers. However, our molecular understanding of the assembly and internal organization of these multicomponent droplets is still incomplete, and rules for the coexistence of condensed phases are lacking. Here, we show that the formation of hierarchically organized multiphase droplets with up to three coexisting layers is a generic phenomenon in mixtures of complex coacervates, which serve as models of charge-driven liquid-liquid phase separated systems. We present simple theoretical guidelines to explain both the emergence and stability of multiphase droplets using the interfacial tension and mean-field interaction parameter as inputs. Coexistence implies differences in macromolecular density, which can be inferred from critical salt concentrations. We show that the coexisting coacervates present distinct chemical environments by concentrating guest molecules in different domains of the multiphase droplets. Our findings suggest that condensate immiscibility may be a very general feature in biological systems, which could be exploited to design self-organized synthetic compartments to control biomolecular processes.

Liquid-liquid phase separation has emerged as an important mechanism for the organization of the intracellular environment.1–3 Membraneless organelles are condensed, often liquid-like bodies formed by phase separation of specific proteins and sometimes also RNA by weak multivalent associative interactions.3–5 They facilitate a wide range of cellular functions, acting as processing bodies, storage granules or organizational hubs.1,6 Various membraneless organelles have hierarchical structures with multiple liquid-like or solid-like inner phases. Examples include nucleoli7 and stress granules,8–10 paraspeckles11 and nuclear speckles.12 The coexistence of multiple condensed phases in a single membraneless organelle has been suggested to reflect the organelles’ complex functions, in which different biomolecular processes take place.
in physically separated regions. However, liquid-liquid phase separation in general, and the emergence of multiphase droplets in cells in particular could also be an inevitable consequence of the underlying molecular interactions. The in vitro reconstituted nucleoli from purified proteins suggest that multiphase droplets could indeed be a generic phenomenon. However, insights into the physical principles and the role of chemical interactions that underlie the emergence and behaviour of multiphase liquid droplets are lacking.

To understand the physical and chemical requirements for liquid-liquid multiphase separation and coexistence, we need to investigate the phase behaviour of well-defined model systems comprising multiple phase separating components, and systematically vary the interactions and composition. A recent simulation study with self-attracting particles as droplet components showed that multiphase droplets can be formed when the interactions between separate droplets components are sufficiently different. However, nearly all components of condensed liquid phases in biological systems are polymeric, such as disordered proteins and RNA, which interact via a limited number of interaction motifs, and it remains unclear how likely it is for these systems to form multiphase droplets. Using an experimental model system of elastin-like polypeptides (ELPs), López and co-workers showed that ELPs with different lower critical solution temperatures could phase separate into multilayered droplets, provided they have dissimilar amino acid content and are sufficiently long. They rationalized their results with a mean-field Flory-Huggins theory for poorly water-soluble polymers, and suggested that subtle changes in amino acid composition might be sufficient to warrant condensate immiscibility and the emergence of multiphase structures. Many biological condensate components differ from ELPs in that they contain a significant fraction of charged residues (e.g., nucleotides, lysines, arginines and phosphorylated amino acids), and there is considerable evidence that charge-driven assembly plays an important role in the formation of various membraneless organelles. We therefore asked if multiple condensates of partially charged macromolecules, such as RNA, could still coexist and what would be their most favourable structural organization.

Here, we show that the formation of multiphase droplets is a generic phenomenon in mixtures of charge-driven liquid-liquid phase separated systems with different critical salt concentrations. We use complex coacervates as model systems for condensed liquid droplets formed by associative phase separation in biological systems. Complex coacervates are liquid droplets (which ultimately coarsen to macroscopically separated phases) that form by associative liquid-liquid phase separation in mixtures of multivalent, oppositely charged molecules. These droplets are enriched in macromolecules, and have been used to model various aspects of membraneless organelles, including their viscoelastic characteristics, controlled formation.
and dissolution by enzymes such as kinases, and salt sensitivity. The phase behaviour of most complex coacervates can be described satisfactorily by a mean-field Flory-Huggins theory, similar to the liquid-liquid phase separation of many components derived from membraneless organelles.

To elucidate the physical and chemical requirements for multiphase separation of charged macromolecules, we prepared complex coacervates with a wide range of chemical charge-bearing groups, inspired by the molecular language of membraneless organelles. While all coacervates form by a similar charge-driven associative phase separation, they are generally not miscible when combined together. Instead, they form core-shell multiphase droplets in which one coacervate is completely engulfed by another. We rationalize the formation of multiphase droplets using mean-field theory of complex coacervates, including the interfacial tension to account for the engulfing of one coacervate droplet by another, and an effective interaction parameter to account for their immiscibility. As in the case of aqueous multiphase systems, small chemical differences between the coacervates, which contribute to non-electrostatic Van der Waals interactions for example, are sufficient to make them immiscible. Differences in coacervate density and critical salt concentration are indicators of those chemical differences, and can be used to predict the formation of multiphase droplets. They translate into differences in interfacial tension, which set the arrangement order inside the multiphase droplets. In general, the densest coacervate phase, which contains the least water, is found at the core of the multiphase droplets. Combinations of polyelectrolytes with high charge densities that form strong ion pairs usually have the highest density. These multiphase coacervate droplets not only offer fundamental insights into the nature of liquid-liquid multiphase separation in biological systems, they also provide design principles for hierarchically organized compartments for advanced release or catalysis.

Results

Multiphase droplets form by mixing different types of complex coacervates.

Many oppositely charged polymers, including oligopeptides and nucleotides, derivatized polysaccharides and synthetic polyelectrolytes, can form spherical coacervate droplets by associative phase separation upon mixing with a preferential 1:1 charge ratio. Over time these droplets coarsen and fuse, ultimately resulting in a bulk liquid coacervate phase. This type of liquid-liquid phase separation (LLPS) of oppositely charged macromolecules is driven by ion pairing and the release of counterions. On first thought, one would expect combinations of two
such complex coacervates to mix and form a single merged coacervate, owing to their common electrostatic interactions.

To investigate what happens to mixtures of complex coacervates, we combined populations of two different complex coacervates (Table S1). As an example, we mixed complex coacervates of single stranded DNA (ssDNA) and trimethylated poly-L-lysine (PLys(Me)$_3$) with coacervates of ssDNA and a lysine-rich ELP, which was fused to GFP (GFP-K$_{72}$).$^{17,32}$ Both combinations form spherical complex coacervate droplets separately (Figure S1) with similar high internal water contents of 80% (w/w) or more at the same salt concentration. Surprisingly, when added together we observed the formation of multiphase coacervate droplets with single or multiple domains of PLys(Me)$_3$/ssDNA inside GFP-K$_{72}$/ssDNA droplets (Figure 1a,b). The PLys(Me)$_3$/ssDNA domains are separated from the GFP-K$_{72}$/ssDNA droplet by a sharp and smooth interface, typical of coexisting liquid phases. ssDNA is the common polyanion of both complex coacervates and is present in both phases, but not in equal concentrations (Figure S1d). We observed similar multiphase droplets for the large majority of coacervate mixtures we tested (Figure 1c-j, Table S2). These structures are reminiscent of multicompartiment membraneless organelles with distinct core domains, such as nucleoli$^7$ and stress granules,$^8$–$^{10}$ and comprise three coexisting liquid phases: the core coacervate, the outer coacervate and the surrounding dilute phase.$^{17}$

Fig. 1. Multiphase complex coacervate droplets formed by mixing different coacervates: (a,b) ssDNA/PLys(Me)$_3$ core coacervates in a ssDNA/GFP-K$_{72}$ outer coacervate phase, viewed in brightfield (a) and confocal fluorescence microscopy (b), (c-j) Same as b, for (c) ATP/PAH cores ATP/PDDA outer phases, (d) PGlu/PAH cores in PGlu/PDDA outer phases, (e) PAA/PLys(Me)$_3$ cores in PAA/GFP-K$_{72}$ outer phases, (f) PSPMA/PAH cores in PSPMA/DEAE-Dex outer phases, (g) S-Dex/PLys(Me)$_3$ cores in S-Dex/GFP-K$_{72}$ outer phases, (h) PSPMA/PAH cores in PSPMA/PDDA outer phases, (i) PSPMA/PDDA cores in PSPMA/Q-Dex outer phases, (j) ATP/PAH cores in PSPMA/PDDA outer phases.
Both the outer droplets and the inner domains are liquids, as demonstrated by their mobility and coalescence (Figure 2b-c, Movie S1-3). Coalescence of core droplets is slow and relatively infrequent as expected based on the high local viscosity inside the corona droplet. The typical timescale of diffusion-limited collision between core droplets is given by 

\[ t_c = \frac{3\eta_s}{4k_B T c_0} \]

where \( \eta_s \) is the viscosity of the surrounding coacervate and \( c_0 \) is the initial number density of core droplets. For a typical outer coacervate viscosity of 100 mPa.s, the average collision time between two core coacervates in a 10 \( \mu \)m outer droplet is of the order of 1 hour. It does not depend on the core coacervate radius, but confinement by the outer droplet may lead faster collision. Interestingly, the very low predicted collision rates may also explain why fusion of certain core domains inside membraneless organelles or condensates in the crowded nucleoplasm is not readily observed, as coarsening occurs over typical timescales of minutes to hours.

When two core coacervate droplets do collide, they fuse driven by a reduction of the total interfacial area, highlighting the fact that they are true liquids in a surrounding liquid (Figure 2b-c, Movie S1-3). The fusion timescale of droplets can be estimated from 

\[ t_f = \frac{\eta_c R}{\gamma_{12}} \]

which only depends on the viscosity of the core droplets (\( \eta_c \)) in this case. Because the core coacervate is typically the densest phase with the highest critical salt concentration (see below), the viscosity of the core coacervate is significantly higher, and fusion of core droplets is much slower than fusion of outer coacervates, in agreement with our observations (Figure 2b-c and Movie S1-3).

To prove that the multiphase droplets we observed are equilibrium liquid-liquid phase separated systems (apart from coarsening), and not kinetic intermediates en route to forming homogeneously mixed coacervates, we prepared the same multiphase droplets by mixing all like-charged components together first without forming separate coacervate populations (Figure S2-3). In that case, we found the same multiphase coacervate droplets with the same core and outer phases (Figure S2-3), indicating that the two coexisting complex coacervates are inherently immiscible and that the multiphase arrangement of phases is energetically favoured.

In order to understand the physical and chemical requirements for liquid-liquid multiphase separation and coexistence, we first analyse the chemical characteristics of the immiscible coacervates. As shown in Figure 1 and Table S2, multiphase droplet formation is not limited to a single type of polycation or polyanion. We can form multiphase droplets with sulfates, phosphates and carboxylates, and with primary, tertiary and quaternary amines. We can use two complex coacervates with a common polyanion (Figure 1), a common polycation (Figure S4), or two polycations and anions (Figure 1j). Finally, even two complex coacervates with a common polyanion and a polycation with the same type of charged group (e.g., primary amine)
can form multiphase droplets when mixed (Figure 1i and S5). However, not all combinations of complex coacervates yielded multiphase droplets. For some combinations with very similar critical salt concentrations, we observed single-phase, mixed coacervate droplets (Table S3 and Figure S6). In brief, complex coacervates with widely varying chemical characteristics can all undergo liquid-liquid phase separation into multiphase droplets, implying a generic explanation underlies this process.

**Multiphase arrangement is governed by interfacial tensions.**

Why do most mixtures of complex coacervates separate into multiphase droplets? To address this question, we consider two aspects of the multiphase droplets. We first discuss why the droplets have a typical multiphase arrangement, as opposed to the individual complex coacervate droplets remaining isolated. Secondly, we discuss why the coacervate phases remain separate and do not mix inside the multiphase droplets.

Fig. 2. Interfacial tension-governed arrangement and fusion in multiphase coacervate droplets. (a) Schematic illustration of four scenarios of two coexisting liquid droplets. (b) Fusion of core PAA/PLys(Me)3 coacervates inside a PAA/GFP-K72 outer phase (cf. Figure 1e). (c) Fusion of PGLu/PDDA coacervates followed by fusion of their internal PGLu/PAH cores (cf. Figure 1d). (d) Engulfing of an ATP/PAH coacervate by a PSPMA/PDDA coacervate (cf. Figure 1j). (e) Dual multiphase arrangement (1/2 and 2/1) in PSPMA/PLys/PLys(Me)3.
The first aspect involves the interfacial energy requirements for the formation of multiphase droplets. Figure 2a shows four possible scenarios for the organization of two droplets of immiscible liquids 1 and 2.\textsuperscript{37,38} a multiphase droplet of 1 in 2 (1/2), a multiphase droplet of 2 in 1 (2/1), a set of attached lenses (1-2) in partial wetting, and complete non-wetting, in which the two droplets remain separate (left of arrow). The latter is a limit of the partial wetting scenario with $\theta \rightarrow 180^\circ$. In experiments, we always observed the complete wetting of one type of coacervate droplets by the other coacervate (1/2 or 2/1), meaning that the core coacervate droplets are spontaneously engulfed by the outer coacervates. We could directly observe the process of engulfing in bright field (Figure 2d, Movie S4) and fluorescence microscopy (Figure S7). It is completely analogous to the wetting-induced formation of double emulsions in microfluidics.\textsuperscript{37}

A coacervate droplet 1 will be engulfed by another coacervate droplet 2 to give 1/2 if the total interfacial energy of the resulting multiphase droplet is lower than the combined interfacial energies of the individual droplets (Figure 2a):\textsuperscript{37} $4\pi R_1^2 \gamma_{1d} + 4\pi R_2^2 \gamma_{2d} > 4\pi (R_1^2 + R_2^2)^{2/3} \gamma_{2d}$, which yields:

$$1/2: \quad \gamma_{1d} > \alpha_2 \gamma_{2d} + \gamma_{12} \quad \text{where} \quad \alpha_2 = \left(1 + \frac{R_2}{R_1}\right)^{2/3} - \left(\frac{R_2}{R_1}\right)^2 \tag{1}$$

The ratio $\alpha_2$ is a measure of the relative droplet size and ranges from 0 ($R_1 << R_2$) to 1 ($R_1 >> R_2$). Based on this balance, the spreading coefficient $S_2$ is defined as: $S_2 = \gamma_{1d} - (\alpha_2 \gamma_{12} + \gamma_{2d})$, and full engulfment of coacervate 1 by 2 requires a positive spreading coefficient ($S_2 > 0$). Likewise, droplet 2 will be engulfed by droplet 1 if:

$$2/1: \quad \gamma_{2d} > \alpha_1 \gamma_{1d} + \gamma_{12} \quad \text{where} \quad \alpha_1 = 1 + (\alpha_2 - 1) \left(\frac{R_1}{R_2}\right)^2 \tag{2}$$

The analysis above predicts that the coacervate with the highest interfacial tension ($\gamma_{1d}$ or $\gamma_{2d}$) is most likely to be engulfed. In the case of complex coacervates, this is typically the densest coacervate with the highest critical salt concentration.\textsuperscript{39–41} It is interesting to note that engulfing depends on the size ratio of the coacervate droplets. Small droplets are always more likely to be engulfed by larger ones. Therefore, both (1) and (2) can be true at the same time in a single system, and 1/2 and 2/1 droplet may be found together if $\gamma_{1d}$ and $\gamma_{2d}$ are nearly identical. We indeed found examples of dual multiphase arrangements in mixtures of poly-L-lysine (PLys) and PLys(Me)$_3$ with poly(3-sulfopropylmethacrylate) (PSPMA) as common polyanion (Figure 2e), with the smaller PLys/PSPMA coacervates engulfed by large PLys(Me)$_3$/PSPMA coacervates (bright regions in darker droplets) and small PLys(Me)$_3$/PSPMA coacervates engulfed by large PLys/PSPMA coacervates (dark regions in brighter droplets). As the droplets coarsen through
coalescence, this arrangement eventually breaks up into one of the two arrangements, depending on the ultimate ratio $\alpha_1$.

Finally, partial wetting is expected for any droplet size ratio ($\alpha_1 \rightarrow 0$ or $\alpha_2 \rightarrow 0$) if

$$1 - 2 \quad \gamma_{12} > \gamma_{1d} \quad \text{and} \quad \gamma_{12} > \gamma_{2d}$$

and the angle $\theta$ between the two contacting droplets (Figure 2a) is given by:

$$\theta = \cos^{-1} \left( \frac{\gamma_{1d}^2 + \gamma_{2d}^2 - \gamma_{12}^2}{2\gamma_{1d}\gamma_{2d}} \right)$$

For very large interfacial tensions between the two coacervates ($\gamma_{12} \geq \gamma_{1d} + \gamma_{2d}$) the angle $\theta$ is equal to $\pi$ (180°) and the droplets become completely nonwetting (i.e., they do not touch and remain isolated). We have not observed any nonwetting or partial wetting for the multiphase coacervate droplets we prepared. This means that the interfacial tension between coacervate phases ($\gamma_{12}$) must typically be smaller than the interfacial tensions of the corresponding individual coacervates ($\gamma_{1d}$ and $\gamma_{2d}$). For these complex coacervate model systems, in which the surrounding liquid is a dilute solution, it is expected that $\gamma_{12}$ is smaller than both $\gamma_{1d}$ and $\gamma_{2d}$, because the difference in density between two coacervates is typically smaller than the difference in density between either coacervate and the dilute phase.\(^{24,29}\) For condensates in biological systems the situation may be different, since the surrounding cytoplasm and nucleoplasm are highly crowded with other macromolecules (i.e., $\gamma_{12} \approx \gamma_{1d} \approx \gamma_{2d}$ or $\gamma_{12} > \gamma_{1d}$). Nonwetting or partial wetting of membraneless organelles could therefore be more common,\(^1\) and be part of the explanation why many membraneless organelles remain separate in the cell.

**Coacervate immiscibility is linked to density differences.**

The second requirement for the formation of multiphase droplets is immiscibility of the two coacervate phases once they are present in the same droplet. Immiscibility is relatively common for solutions of long water-soluble polymers, such as PEG, polyacrylamide and dextran,\(^{43}\) and the phase behaviour of the resulting aqueous multiphase systems can usually be rationalized using a mean-field Flory-Huggins theoretical framework.\(^{17,44}\) The Flory interaction parameter $\chi$ provides a measure of the strength of interaction between different components in a mixture, relative to their self-interaction (high positive values reflect more unfavourable interactions). Beyond a critical $\chi_c$, a mixture of two components phase separates. This critical value depends strongly on the length of the coexisting components, as translational entropy becomes negligible for long polymers:
\[ \chi_c = \frac{1}{2} \left( \frac{1}{\sqrt{N_1}} + \frac{1}{\sqrt{N_2}} \right)^2 \]  

where \( N_1 \) and \( N_2 \) are the chain lengths of the two species. For two long polymers, phase separation already occurs near \( \chi_c = 0 \), depending slightly on the presence of a common solvent such as water in the case of aqueous multiphase systems. In other words, even very small differences in chemical characteristics already result in sufficiently unfavourable interactions to cause demixing of long polymer solutions.\(^{43}\)

A similar Flory-Huggins formalism can be used to describe the phase separation of oppositely charged macromolecules at 1:1 charge ratio, by using an effective Flory interaction parameter that depends on the ionic strength (Supplementary Information).\(^{4,19,24,29,45}\) To extend this framework to predict the miscibility of multiple coacervates, we express the effective interaction parameter \( \chi_{12} \), quantifying the interaction strength between the two coacervates,\(^{17}\) in terms of the interaction parameters that characterize the two separate coacervates and their coexisting dilute solution, \( \chi_{1d} \) and \( \chi_{2d} \) (Supplementary Information): \( \chi_{12} \approx \frac{1}{2} (\chi_{1d} - \chi_{2d})^2.\)\(^{46}\) For the interaction between coacervates \( \chi_{12} \) to exceed the critical value \( \chi_c \), the two coacervates should have sufficiently different effective interaction parameters \( \chi_{1d}, \chi_{2d} \). We can estimate the interaction parameters \( \chi_{1d} \) and \( \chi_{2d} \) from the critical salt concentrations of the complex coacervates. The interaction parameter is linked to the polymer density of the coacervate phase and to the interfacial tension (Supplementary Information). A high polymer density is a direct indication of a large \( \chi \) characterizing the interactions that underlie phase separation. For complex coacervates, this interaction strength and the coacervate density is tuned by the salt concentration.\(^{24,29}\) Above a critical salt concentration, the coacervates are completely soluble (i.e., \( \chi < \chi_c \)) and lower salt concentrations correspond to stronger demixing.\(^{29,30}\) When two different complex coacervates are added together at the same salt concentration, the coacervate with the highest critical salt concentration will have the highest density (Figure 3a), and interfacial tension. This implies that two coacervates with significantly different critical salt concentrations, expressed by different \( \chi \) parameters, will have different densities, and their mutual interaction parameter \( \chi_{12} \) is expected to be sufficiently large to warrant immiscibility.

We tested this theory by measuring the critical salt concentrations of all complex coacervates we used (Table S3), and found that the critical salt concentrations of all combinations that was indeed significantly different (>10%). By contrast, when we mixed various coacervates with very similar critical salt concentrations, we found single-phase, mixed coacervates (Figure S6), in excellent agreement with our predictions. Our theory also explains why generally complex coacervates of primary and quaternized amines with the same polyanion yield multiphase droplets. Most primary polyamines have significantly higher critical salt
concentrations than the corresponding tertiary or quaternized amines (Table S3), owing to their stronger ion pairing with most negatively charged groups.\textsuperscript{47} The primary polyamine coacervates therefore have higher densities and higher interfacial tensions than most quaternized amine coacervates, and they usually end up in the core of a multiphase droplet. An exception is the combination of the primary amine containing GFP-K\textsubscript{72} and quaternized amines, such as PLys(Me), with a common polyanion, such as poly(acrylic acid) (PAA) or dextran sulfate (Figure 1e, g). GFP-K\textsubscript{72} is consistently found to be the outer coacervate phase, because it has a much lower charge density than trimethylated polylysine and therefore a lower effective interaction parameter and density (Supplementary Information).

Fig. 3. Immiscible coacervates have different critical salt concentrations and coacervate densities. (a) Schematic phase diagram of complex coacervation at charge neutrality showing binodal curves for coacervates with increasing interaction strength, originating for example from increasing hydrophobicity. (b) Step-wise dissolution of PSPMA/PAH/PDDA multiphase droplets, shown by confocal fluorescence microscopy (top row) and bright field (bottom row).

The molecular origin of complex coacervate immiscibility, and by extension, of differences in critical salt concentration is the residual interaction between polymers apart from
electrostatics (e.g., Van der Waals). It has previously been reported that small ionic groups, such as primary amines bind more strongly than bulkier quaternary amines, and that more hydrophilic charged groups bind more weakly, which agrees well with our critical salt concentrations. In the field of polyelectrolyte multilayers, these insights are commonly applied to control the strength of layer attachment. Applied to multiphase droplets, it means that a complex coacervate with a higher critical salt concentration must be more hydrophobic than one with a lower critical salt concentration, and therefore have a higher density and end up in the core of multiphase droplets. In addition, more flexible polymers are also expected to form complexes more effectively without requiring bending energy, and therefore have higher critical salt concentrations. In the context of biological systems, our theory suggests that most condensate components will not mix, since these are typically long molecules and small variations in amino acid composition can already result in sufficiently unfavourable interactions, in agreement with previous findings. Moreover, strongly interacting components will likely give rise to higher densities and end up at the core of hierarchically organized organelles, provided the interfacial tension permits engulfing (Figure 2a).

**Multiphase complex coacervates can be selectively dissolved.**

We can take advantage of the fact that the coexisting phases in all multiphase droplets have different critical salt concentrations by selectively dissolving or condensing the outermost coacervate phase. Figure 3b shows that multiphase coacervates with a poly(allylamine hydrochloride) (PAH)/PSPMA core and a poly(diallyl dimethylammonium chloride) (PDDA)/PSPMA corona phase can be dissolved in a step-wise fashion. Upon increasing the salt concentration from 0.5 M to 1.5 M, the corona phase is dissolved, while the core coacervates remain intact up to 3.0 M. These steps can be reversed again to form the same multiphase droplets as in Figure 1h in a sequential manner (Figure S8).

**Differential partitioning in coexisting coacervate phases.**

Complex coacervates are known for their ability to sequester a wide range of guest molecules, depending on their chemical characteristics. As the coexisting coacervates in multiphase droplets differ in density and hydrophobicity (see above), they are expected to take up guest molecules to different extents. We investigated the partitioning of a range of small guest molecules with different charge and hydrophobicity in multiphase droplets of PSPMA/PAH/PDDA (Figure 1h).
As shown in Figure 4, all guest molecules were concentrated in the multiphase droplets ($K_1>1$, see Methods) with partition coefficients ranging from 1.1-1.2 for neutral polar molecules such as eGFP to 5.3-15 for relatively hydrophobic dyes such as ThT (Figure S9). Inside the multiphase droplets most guests concentrated in the more hydrophobic PAH/PSPMA core droplets ($K_2>1$). Hydrophobic and zwitterionic molecules (ThT, 6-aminofluorescein, rhodamine B, Nile red) showed the strongest fluorescence in core coacervates relative to the outer coacervate phase. We note that this increase in fluorescence can originate from both a higher dye concentration, and an increased fluorescence quantum yield due to enhanced dimer or H-aggregate dissociation (e.g., rhodamine B and Nile red)$^{52}$ or reduced rotational freedom (e.g., ThT)$^{53}$. Neutral and negatively charged molecules (eGFP, TCPP, carboxyfluorescein) showed the weakest increase in fluorescence in the core coacervates. Finally, fluorescein-labelled PEG was concentrated in the core of PDDA/Q-Dex/PSPMA multiphase droplets, which is depleted of dextran, as expected based on the ability of PEG and dextran to phase separate.$^{43}$

Interestingly, some relatively hydrophobic guest molecules with a high net charge strongly adsorbed to the interface between the two coacervate phases. Both SYBR Gold (SG) and methyl blue (MB) showed strong fluorescence localized in a ring around each of the core droplets (Figure 4i-j). We attribute this adsorption to the amphiphilic nature of these guest molecules: their hydrophobic core nature favours concentration in the dense PAH/PSPMA core coacervate, while their hydrophilic, charged moieties favour accumulation in the more hydrophilic PDDA/PSPMA shell. A similar accumulation at the coacervate interface has been observed before for molecules with an amphiphilic nature.$^{54,55}$ It would be interesting to see if a
similar mechanism could result in accumulation of specific biomolecules at the interface between domains in the nucleolus, for example.\textsuperscript{7}

**Hierarchical organization in three-phase droplets.**

Our theory is not limited to multiphase droplets of two coexisting coacervates. Like in the case of aqueous multiphase systems, many water-based complex coacervates can in principle coexist.\textsuperscript{43} As many coacervates have different critical salt concentrations (Table S3), we expect that multiphase droplets with three or more coacervates could also be formed, either as hierarchical core-shell droplets, in which the core coacervate is embedded in an intermediate coacervate phase, which is embedded in an outer coacervate phase, or as multiple loose cores in a common outer coacervate phase. The relative magnitudes of the different coacervate-coacervate interfacial tensions will determine which scenario corresponds to the lowest surface energy. Examples of hierarchical arrangement have been found in the case of nucleoli in living cells,\textsuperscript{7} and in ELP droplets in vitro.\textsuperscript{17}

To demonstrate that mixtures of complex coacervates can also form multiphase coacervates, we prepared three combinations of three different coacervates, one with five components in total (two polycations and three polyanions) and two with a common polyanion in all coacervates. All combinations yielded hierarchical three-phase droplets (Figure 5), in which all coacervates are completely wetted by the shell phase surrounding them. Fusion between domains can be observed at all levels, illustrating the liquid nature of all coexisting phases (Movie S5).

![Fig. 5. Multiphase complex coacervate droplets with three coexisting condensed phases. (a,b) An ATP/PAH inner core, surrounded by a PSPMA/PDDA shell in a PAA/PDDA outer coacervate phase, visualized in brightfield and by confocal fluorescence microscopy (PSPMA-Fi fluorescence). Note that (a) and (b) do not show the same position. (c,d) PSPMA/PAH inner core, surrounded by a PSPMA/PDDA shell in a PSPMA/Q-Dex coacervate phase, visualized at the same position in brightfield (c) and by confocal fluorescence microscopy (d, PSPMA-Fi fluorescence, see Figure S10 for an overlay).](image-url)
Conclusions

We have shown that a wide range of complex coacervates are immiscible and give rise to the formation of multiphase droplets in which multiple condensed liquid phases coexist. A multilayer arrangement is favoured if the coacervate-coacervate interfacial tension is lower than the interfacial tension of one of the coacervates with the surrounding dilute phase. Inside a multiphase droplet, coacervates are likely to remain demixed if they have dissimilar densities, which can be inferred from differences in critical salt concentration. The densest coacervate is the most hydrophobic and typically found at the core of the multiphase droplets. Guest molecules can distribute over all coexisting phases and become concentrated in one of the coacervates.

Our findings show that condensate immiscibility may be a very general feature in biological systems, as even condensates formed by the same attractive interactions between opposite charges do not mix when the components are sufficiently long. Our systematic analysis using model systems, supported by simple theoretical arguments, offers guidelines for understanding the physical and chemical requirements for liquid-liquid multiphase separation and coexistence. Moreover, our ability to predict and control these hierarchical multiphase complex coacervate droplets opens new ways to design smart self-organized compartments for controlled storage, catalytic conversion and release of bioactive molecules.

Methods

Materials.
All polymers used in this study are commercially available or synthesized according to previously reported methods. An extensive list of all polymers and their characteristics can be found in the Supplementary Information. Salts, including sodium chloride (NaCl), magnesium chloride (MgCl₂·6H₂O), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and adenosine triphosphate disodium salt (ATP) were purchased from Sigma. All solutions were prepared in Milli-Q water (MQ) and the pH of buffer solutions was adjusted using sodium hydroxide (NaOH, Baker) and hydrochloric acid (HCl 37%, VWR). mPEG-trimethoxysilane (5 kDa) was purchased from Jenkem Technology. Dye molecules for partitioning experiments were dissolved in Milli-Q (pH 7) or dimethyl sulfoxide (DMSO, Sigma) before use at the following concentrations. Rhodamine B (RhoB, 1.5 mM in MQ), Thioflavin T (ThT, 1.0 mM in MQ), 6-Aminofluorescein (6-AF, 20 mM in MQ), 5(6)-carboxyfluorescein (5(6)-CF, 8.5 mM in DMSO), 4,4′,4″,4‴-(porphine-5,10,15,20-tetrayl)-tetrakis(benzoic acid) (TCPP, 2.0 mM in MQ), Methyl blue (MB, 3.1 mM in MQ) and Nile Red (NR, 31 mM in DMSO) were purchased from Sigma. PEG-difluorescein (8 kDa, 150 mg/mL) was purchased from Chemicell and diluted to 30× before use. SYBR gold nucleic acid stain (10,000× concentrate in DMSO) was purchased from Thermo Fischer and diluted 100× before use. eGFP (84.7 µM) was produced and purified using a custom-made IVTT protocol as described elsewhere.56
Complex coacervate preparation.
Stock solutions of all polymers, salts and buffers were prepared at the indicated concentrations in Milli-Q water. Typically, polymer stock solutions were prepared at 50 mg/mL, pH 7 without added salt.

Single-phase coacervates were prepared by first mixing NaCl (3.0 M stock), HEPES (0.50 M stock) and Milli-Q water in a microcentrifuge tube (0.5 ml, Eppendorf). To the mixture, 1:1 charge-stoichiometric quantities of the positively and negatively charged polymers or molecules were added to a total volume of 20 µL. The final NaCl concentration in the mixture varied from 6 mM to 1.0 M (Table S4). Mixing was done by gentle pipetting (3×) before each measurement. To the coacervates containing ATP and ssDNA, 5 mM MgCl₂ was added.

For preparation of multiphase coacervates, we used two methods. For the first method, we mixed all the like-charged polymers together and added those at a 1:1 stoichiometric ratio to the premixed buffer and salt solutions (Figure S2). For the second method, two different types of coacervates were prepared separately, as described above, diluted to the same salt concentration, and then mixed together in a separate tube (Figure S3). Both methods yielded the same multiphase droplets.

For the three-phases coacervates, three samples were prepared, (1) PSPMA/PDDA + ATP/PAH + PAA/PDDA, (2) PSPMA/PAH/PDDA/Q-Dex and (3) PSPMA/PAH/PDDA/DEAE-Dex. All samples were prepared by mixing three separately prepared coacervates after dilution to the same salt concentration (0.3 M for sample 1, 0.2 M for sample 2 and 0.4 M for sample 3), as described above. Mixing was done by gentle pipetting (3×) before each measurement.

Widefield and confocal microscopy.
Images were obtained using a CSU X-1 Yokogawa spinning disc confocal unit connected to an Olympus IX81 inverted microscope, using a 100x oil immersion objective (NA 1.5) and recorded on an Andor iXon EM-CCD camera. For imaging, a 10-30 µL aliquot of a coacervate mixture was added to a custom-made mPEG-silane passivated PDMS observation chamber on a cover glass slide (No 1.5). PDMS chambers were prepared by curing a slab of PDMS (Sylgard 184 elastomer kit, 10:1 PDMS:crosslinker) for 90 minutes at 65°C, cutting out 5×5 mm square wells, and bonding them to a cover glass using plasma activation. The PDMS and glass surfaces were PEGylated after plasma activation by immersing them in a 30 mg/mL solution of mPEG-silane (5 kDa) in ethanol for 2 hours at 60°C.

Critical salt concentrations.
The critical salt concentration of single-phase coacervates was measured on a microplate reader (Tecan Spark), equipped with a microinjector, as described elsewhere. Briefly, turbidity of a coacervate solution with a total starting volume of 50 µL above the critical salt concentration was monitored as a function of the concentration of NaCl at a wavelength of 600 nm and a temperature of 26 ± 1°C in 96-well plates (Greiner Bio-one, clear flat-bottom wells) by dilution with MQ in 5 µL steps. Samples were shaken for 0.3 s before every readout. The critical point was determined by extrapolating the first-order derivative at the inflection point to zero turbidity. Note that this critical salt concentration does not take into account ions from other sources than the added NaCl, and the actual critical ionic strength may be slightly higher.

Selective coacervate dissolution and condensation.
For the selective dissolution and condensation of multiphase droplets, we selected PSPMA/PDDA/PAH and prepared the sample according to method 2. For selective dissolution, we started at a NaCl concentration of 0.50 M and added 20 µL of the mixture into a large-volume mPEG-silane modified PDMS sample chamber on a cover glass slide. We then added increasing amounts of NaCl from a 4.0 M stock to reach the indicated salt concentrations, mixed the sample by gentle pipetting (3×) and recorded
images. For the reverse experiment of selective condensation, we prepared the mixture at a NaCl concentration of 3.0 M and added 20 µL of the mixture into a large-volume mPEG-silane modified PDMS sample chamber on a cover glass slide. We then added increasing amounts of Milli-Q to decrease the NaCl concentration to the indicated values, mixed the sample by gentle pipetting (3×) and recorded images.

**Partitioning experiments**

For partitioning experiments 20 µL aliquots of a selected multiphase coacervate system (PSPMA/PDDA/PAH or PSPMA/PDDA/Q-Dex) were added to neighbouring mPEG-silane modified PDMS sample chambers on a cover glass slide. Small quantities of the stock solutions of the dye molecules were added to the multiphase coacervate droplets, mixed by gentle pipetting, and visualized by excitation at the indicated wavelengths. TCPP (0.3 µL) and ThT (2 µL) were excited at 405 nm. 6-AF (0.2 µL), 5(6)-CF (0.2 µL), PEG-di-fluorescein (0.2 µL), SG (1 µL) and eGFP (2 µL) were all excited at 488 nm. RhoB (1 µL) and NR (0.2 µL) were excited at 561 nm. Finally, MB (0.4 µL) was excited at 640 nm. The partition coefficient \( K \) was determined from average fluorescence intensities as

\[
K_1 = \frac{I_{c.out} - I_b}{I_d - I_b}
\]

and

\[
K_2 = \frac{I_{c.in} - I_b}{I_{c.out} - I_b},
\]

where \( I_b \), \( I_d \), \( I_{c.out} \) and \( I_{c.in} \) are the intensity of a blank solution, the dilute phase surrounding the multiphase droplets, the outer coacervate layer and the inner core coacervate of the multiphase droplets, respectively.

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Author contributions
E.S. conceived and designed the experiments. T.L. performed all experiments and analyzed the data. E.S. and T.L. wrote the manuscript.

Additional information
Supplementary information containing extended methods, supplementary equations, additional figures and movies is available.

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