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Liquid-liquid phase separation during amphiphilic self-assembly

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The self-assembly of amphiphilic molecules in solution is a ubiquitous process in both natural and synthetic systems. The ability to effectively control the structure and properties of these systems is essential for tuning the quality of their functionality in biomedicine, food science, separation science and templated synthesis. Here we propose a new model for amphiphilic self-assembly which describes how self-assembly can be preceded by liquid-liquid phase separation. Analysis of a model block copolymer system by a combination of theory, Liquid Phase Electron Microscopy and Self Consistent Field computations reveals that these liquid phase precursors can play a role in determining the structural properties of vesicles such as their size, membrane thickness and the onset of kinetic traps during self-assembly. We anticipate that this new insight will allow the design of self-assembled structures by creating models which consider and utilize liquid phase precursors.

Amphiphilic molecules self-organize in selective solvents, forming a wide range of higher order structures. In biological systems the assembly of amphiphilic molecules results in the formation of cell membranes, transport vehicles and reaction vessels with precisely controlled size and contents. In synthetic systems, surfactants, phospholipids and block copolymers form a variety of amphiphilic assemblies such as micelles, vesicles, toroidal and bicontinuous structures that are used for many different applications¹⁻³. To design specific structures, and to achieve the precise control often observed in nature, knowledge of the thermodynamic and kinetic landscapes of the assembly process is required. Despite significant efforts,⁴⁻⁹ the molecular mechanisms underlying the structural evolution in amphiphilic self-assembly are still not fully resolved, particularly in relation to controlling out-of-equilibrium pathways.^{10,11}

Amphiphilic assemblies can be prepared by either top-down or bottom-up methods. Top-down methods can be used for relatively soluble or mobile molecules where a precursor phase is

broken down and dispersed in solution, e.g. in the direct dissolution of powders and thin film rehydration.¹² In bottom-up methods, molecules are first dissolved in a good solvent after which a change in the solution conditions or the molecular structure increases amphiphilicity and initiates self-assembly. Examples are the ethanol injection of lipids¹³, and many strategies for block copolymer self-assembly, including the solvent switch method¹⁴, temperature¹⁵ and pH induced¹⁶ aggregation, and polymerization induced self-assembly (PISA)¹⁷⁻¹⁹.

For the bottom-up assembly of block copolymer systems the first step in the self-assembly process is thought to be the formation of spherical micelles^{6,17}, which may either stabilize or transform into other structures, such as cylinders or vesicles^{6,17}. It is well-known that under specific conditions homopolymer solutions can undergo liquid-liquid phase separation, forming polymer rich liquid droplets as a result of a spinodal decomposition process^{20,21}. Recently, Sato and Takahashi described how phase separation can occur in amphiphilic copolymer systems, under weakly amphiphilic conditions²². As bottom-up self-assembly involves the gradual increase of amphiphilicity by changing the solvency of a part of the molecules involved, we reasoned that liquid-liquid phase separation could be a precursor to self-assembly for many systems. Moreover, it has been shown that when self-assembly occurs in liquids with phase separated domains,²³ these domains will influence the assembly process, hence, these liquid phase precursors may be important for controlling the final self-assembled structures.

Here, we investigated this phenomena by modifying the recently developed theoretical framework of Sato and Takahashi²² (see Methods in Supplementary Information) to demonstrate that phase separation should occur during bottom-up self-assembly in a wide range of amphiphilic systems. To prove this theory we investigate a model vesicle forming system, poly(ethylene oxide)-block-poly(caprolactone) (PEO-*b*-PCL).²⁴⁻²⁸ Using a combination of liquid phase transmission electron microscopy (LP-TEM),²⁹⁻³¹ and self-consistent mean field (SCF) theory,³²

we show the formation of the liquid precursor phase and reveal the role of phase separation in the vesicle formation process.

Fig. 1. Phase diagrams for generic amphiphile A_N-B_M , showing how the assembly pathway from soluble unimers (S) to a liquid-liquid phase separation (LL) and micellization (M) varies with different parameters. a. variation with the fraction B groups per polymer f_B for highly soluble A groups. b. variation with f_B for moderately soluble A groups. c. variation with χ_{AB} for moderately soluble A groups. Example values are shown for common block copolymers, the dashed blue line represents the critical value of χ_{AB} used to build the phase diagram d. variation with total chain length ($N_A + N_B$) for moderately soluble A groups. d) schematic representation of the three phases reported in the phase diagram: the homogeneous polymer solution (S), the phase-separated polymer rich liquid droplet (LL) and the micelle (M), which represent the starting point in the self-organization process.

Results and discussion

Liquid-liquid phase separation in amphiphilic self-assembly. We use a simple model to describe the self-assembly process of a generic amphiphile (A-B) in a solvent (S) with a set of interaction parameters (χ_{AS} , χ_{BS} and χ_{AB}). A and B are the solvophilic and solvophobic moieties where N_A and N_B are the number of repeating units respectively, and χ are the Flory-Huggins interaction parameters between the different pairs of components.

We developed a modified expression for the free energy of mixing for the copolymer (See Methods in Supplementary Information) and compared it with the free energy of mixing for the micelle phase²². The pathway of bottom-up self-assembly is modeled by the gradual increase of the interaction parameter between the solvophobic block and the solvent (χ_{BS}) (Fig. 1), or the increase in both χ_{BS} and N_B ^{18,19}. For highly soluble A groups (Fig. 1a, $\chi_{AS} = 0.1$) we obtain a direct transition from soluble unimers to micelles. However, for moderately soluble A groups ($0.2 < \chi_{AS} < 0.5$),²² – as for most non-ionic polymers dissolved in water and most polymers dissolved in organic solvents³³ – the model predicts a transition from soluble unimers to a liquid-liquid phase separated state before self-assembly occurs (Fig. 1b, $\chi_{AS} = 0.45$). This liquid precursor is predicted to form, regardless of the fraction of B groups per polymer, f_B (Fig. 1b), for a wide range of compositions (Fig. 1c), and for all amphiphiles with total number of repeating units < 350 (Fig. 1d). Hence this polymer-rich liquid phase is expected to act as a precursor in the bottom-up formation of spherical micelles, cylindrical micelles and vesicles from a wide variety of amphiphilic systems (Fig. 1d). This process is somewhat analogous to the liquid phase precursors found in studies of protein, biomineral and inorganic crystal formation – where the formation of the precursors is known to lower activation barriers to crystallization and can result, for example, in increased nucleation rates.³⁴⁻³⁹ These formation processes are often described as two-step formation pathways, where the liquid phase precursors are meta-stable intermediate species with respect to the crystalline products. It is important to note that in the present case the precursors are

the most thermodynamically stable states under the conditions at that point during the synthesis, and they act as precursors to amorphous self-assembled materials.

To confirm the existence of this polymer rich liquid phase and to understand its influence on self-assembly we investigate –with theory, and experiment - the bottom-up formation of vesicles from PEO-*b*-PCL,^{27,28,40,41} a polymer widely studied for use in biomedical applications^{24,42}. We consider the formation of vesicles of PEO_{2K}-*b*-PCL_{10K} through the solvent switch process from acetone (good solvent) to water (selective solvent) in which micelles are considered the first type of assemblies to appear^{19,21,36}. Using our model we derive the critical compositions at which phase separated droplets ($\phi_W^D = 0.05$) and self-assembled micelles ($\phi_W^M = 0.11$) become stable, indicating that liquid-liquid phase separation is stable for $0.05 < \phi_W < 0.11$, after which self-assembly (micelle formation) is expected to occur (Supplementary Fig. 1).

Fig. 2. Formation process of PEO_{2K}-b-PCL_{10K} block copolymer vesicles by LPEM. a, single frame time series from Supplementary Movie 1. b, comparison of the most mature vesicle in the LPEM movie with a typical vesicle from the bulk self-assembly analyzed ex-situ by cryoTEM. c, analysis of a single vesicle is displayed as a relative intensity cross-sectional time series, insets show the first and last frame with the vertical black lines indicating where the cross-section was taken. d Radial average intensity plots for the vesicle. The dashed black vertical line in c and d, located at 137 s, indicates the transition from the phase separation regime to the self-assembly regime, when the membrane of the vesicle starts to form. Scale Bars a: 400 nm, c: 200 nm.

Direct observation of liquid phase precursor. To directly observe this polymer rich liquid phase precursor and investigate its effect on the formation of the vesicles we monitor the assembly process in real-time using liquid phase transmission electron microscopy (LP-TEM)^{29,31,35,43-46}. *In-situ* observations were made using a liquid flow cell holder, which allows for solvent mixing inside the cell (see Methods and Supplementary Fig. 2-4). It is important to note that block copolymer self-assembly processes have both thermodynamic and kinetic components meaning that the resulting morphology can be highly dependent on the preparation conditions.^{12,17,41,47,48} As controlling solution mixing inside the liquid cell is challenging, vesicle formation was achieved through multiple mixing experiments, where vesicles were successfully formed on four occasions out of > 50 attempts. Here we focus our analysis on the formation of the liquid phase precursors and their influence on the formation of the vesicles, however, other morphologies were observed in both the in-situ and ex-situ experiments, data shown in the supplementary information. Self-assembly of the vesicles was recorded with a 1 second integration time and a controlled, low electron flux of $30 \text{ e} \cdot \text{nm}^{-2} \cdot \text{s}^{-1}$ ^{46,49}. Over a period of 900 seconds, as the water content increases in the cell and χ_{BS} increases (see Equation (6) in Methods), we observe the formation of block copolymer vesicles of ca. 100-200 nm in final diameter (Fig. 2a, Supplementary Fig. 5, Supplementary Movie 1). During vesicle formation, we observe that there is little movement of the individual objects, indicating adherence to one of the silicon nitride membranes enclosing the liquid cell. This is a common feature in liquid cell TEM experiments⁴⁴, which reduces motion blur of moving objects, allowing for more accurate quantitative analysis of individual particles. For LPEM the effects of the electron beam and confinement within the cell must be considered,⁴⁹⁻⁵² a detailed discussion, as well as control experiments are given in the supplementary information. Briefly, scanning electron microscopy (SEM) was performed on the chips after disassembly of the cell and drying, which shows the spherical nature of the particles formed in the cell, as well as the formation of particles far away from the viewing area, i.e. with no effect from the electron beam (Supplementary Fig. 6). Furthermore, comparison with the cryoTEM analysis of self-assembled structures prepared ex-situ shows that the final particle diameter and membrane thickness are

comparable to the vesicles formed in the lab, indicating particle adherence to the silicon nitride window does not greatly influence the final morphology (Fig. 2b, Supplementary Fig. 7 and 8 and Methods).

The data analysis for interpreting the self-assembly of individual vesicles was implemented by in house scripts programmed in Matlab (Supplementary Fig. 9-17, supplementary information and Supplementary Movie 2 and 3). The formation process of single vesicles can be displayed as a series of central line intensity profiles vs time, herein referred to as a cross-sectional time series (Fig. 2c, see Supplementary Fig. 11). The cross-sectional times series shows an increase of contrast throughout the evolution of the vesicle, from a highly solvated state with extremely low contrast ($t < 137$ s), to a desolvated state where the PCL membrane displays high contrast ($t > 137$ s) (see supplementary information). The size and contrast of the highly solvate state strongly suggest it is not a self-assembled structure, but that we are indeed capturing the structure of the polymer rich liquid phase precursor. The radial averaged intensity maps (Fig. 2d, 2e and Supplementary Fig. 15) quantify the contrast increase in time and show that the membrane thickness increases over time until a plateau value is reached.

Fig. 3. Time-lapsed relative intensity maps show the evolution of the membrane for vesicle 1. a, b, relative intensity angular maps (b) and corresponding images (a) showing the vesicle membrane evolves from the dense liquid phase into a continuous structure through an inhomogeneous process. The black arrow indicates the dense liquid phase ($t = 62$ s), the white dashed circles indicate the initial formation of the membrane patches ($t = 125$ s) and the white dashed boxes indicates the closure of the patches to form a continuous membrane, and the concomitant depletion of the dense liquid phase ($352 < t < 689$ s), scale bars = 100 nm.

To understand the vesicle formation pathway we also analyse the local structure of the membrane by plotting relative intensity maps as a function of angular location around the vesicle (Fig. 3, Supplementary Fig. 17 and supplementary information for more details). The angular map time series (Fig. 3b) shows the spherical morphology of the liquid phase precursor ($t = 62$ s). The

time series further shows that the membrane, corresponding to the darker regions in the intensity scale, forms through the appearance of multiple separate patches ($t = 242$ s), at the interface between the liquid precursor and the bulk solution. These patches then grow and coalesce to form the continuous membrane (Fig. 3a, $t = 689$ seconds).

Fig. 4. Overview of the vesicle formation mechanism. Comparison of a, individual frame time series with b, 30 frame averages time series (scale bar = 100 nm) and c, corresponding plot profiles for the 30 frame averages. The individual frame at $t = 46$ s shows separate dense patches. The 30 frame average over $25 < t$

< 62 s shows these dense patches are localised inside a spherical object (the polymer-rich droplet). The 30 frame averages at $102 < t < 137$ show that the droplet has internally phase separated creating a membrane like structure. At $t = 242$ s, the individual frame shows an ‘open vesicle’ structure, whereas the 30 frame average over this time period indicates the phase separation within the dense liquid phase has increased. At $880 < t < 912$ seconds the single frame and 30 frame averages are comparable, indicating a stable structure has formed. d, The schematic combines the short and long time domain images into a single mechanistic diagram to describe the formation process of the vesicles.

To provide further experimental evidence for the polymer-rich liquid droplets and show their role in the overall formation process we consider the evolution of the vesicles over both short and long time domains (Fig. 4). As pixel contrast in the TEM images is directly related to the accumulation of polymer (see supplementary information), individual frames show the location of the assemblies at a single time point (Fig. 4a), whereas images created by averaging 30 frames show the localisation of the assemblies within the liquid phase precursor (Fig. 4b, c, supplementary information for more details and Supplementary Fig. 18 and 19.). The single frame time series shows that in the early stages ($t = 46$ s) only a small number of individual pixels display contrast. Over time we see the appearance of several distinct assemblies ($t = 130$ s), which grow and coalesce ($t = 242$ s) to eventually form a stable vesicle structure ($t = 912$ s). The 30 frame average time series shows that in the early stages ($25 < t < 62$ s) the precursor has a spherical morphology. Over time the nucleation and growth of the assemblies at the interface appear as a continuous structure when analysed in the long time domain, giving the appearance of an ‘internal phase separation’ process ($102 < t < 137$ and $209 < t < 242$ s). In the final stages ($880 < t < 912$ s) the single frame and 30 frame averages are comparable, indicating a stable structure has formed. This shows that the time-domain in which the in-situ data is displayed can change the apparent formation mechanism.

Since the resolution of the LP-TEM movie is not sufficient to analyse the early stages of the assemblies, we performed Scheutjens-Fleer SCF computations to determine the equilibrium organization at different solvent compositions (see Methods and Supplementary Fig. 20-22, Tables 1 and 2)³². This method is based on a Flory-Huggins(FH)-like lattice theory²⁰, using FH interaction parameters (χ)³³, and a small systems thermodynamics approach⁵³. The SCF method goes beyond

FH in the sense that it enables to account for inhomogeneities (concentration gradients) of all compounds allowing the description of self-assembly, e.g. by predicting the size and morphology PEO-*b*-PCL assemblies in water^{41,54}. For PEO_{2K}-*b*-PCL_{10K} the SCF computations predict that the assemblies should first form spherical micelles at $\phi_w = 0.1$, which convert to cylindrical micelles at $\phi_w = 0.11$ and then form a bilayer structure at $\phi_w = 0.12$. It is important to note that the SCF simulations of self-assembly are in good agreement with our theoretical predictions that micelle formation occurs at $\phi_w = 0.11$. Therefore, we propose that the formation of polymer vesicles occurs through the nucleation and growth of a polymer-rich liquid droplet followed by the localised assembly of spherical micelles at the interface. These micelles then grow by unimer addition and coalescence, evolving into cylinders and bilayers eventually closing to form vesicles (Fig. 4d). As described above this process is somewhat analogous to the two-step nucleation processes observed in the study of crystal formation,³⁴⁻³⁹ but with the specific challenge to determine the location of an amorphous-amorphous interface. Here, we define the interface using intensity thresholding, as the increase in intensity is related to the desolvation of the particle (see Supplementary Information). Moreover, as we show in Figure 4, the time domain over which the data is analysed has an influence on the observed intensity distribution. However, regardless of the actual threshold set, the non-homogenous membrane formation process described in Figure 3 is present, indicating that multiple separate ‘patches’ are localized within an area that the vesicle eventually forms. This observation was also made when repeating the in-situ vesicle formation process (Supplementary Figures 27, 28, 29 and 30), where the dynamic patches are even more clearly visible (Movies S8 and S9). However, in this case there is a significant increase in particle diameter in conjunction with the formation of the higher contrast self-assembled structures. This is likely due to slight differences in mixing rates during the LPEM experiment which cannot be precisely controlled. Furthermore, it indicates that the kinetics of how the phase diagram (Figure 1) is traversed can affect the formation mechanism. In another repeat of the in-situ formation process (Supplementary Movie 10, Supplementary Figures 31 and 32) the patch mediated process is not directly observable in the movie. This could be due to faster dynamics of the assemblies

resulting in an insufficient temporal resolution to observe them as individual objects. Interestingly, the post-mortem analysis of these chips revealed a combination of vesicles and multi-compartment vesicles (Supplementary Figure 31). The cross sectional time series and angular maps show a growth and internal phase separation mechanism, however the angular map at $t = 80$ seconds does show an inhomogeneous initial structure (Supplementary Figures 32.) Further work is needed to determine the how variation in the nucleation and growth rates of the polymer-rich liquid droplets and self-assembled species can influence the final particle morphology.

Fig. 5. Quantitative analysis of the LPEM data and comparison with SCF simulations. Evolution of a, particle diameter, D , b, membrane thickness, T , and c, diameter-normalized membrane contrast, C/D with time for 5 vesicles from the LPEM Supplementary Movie 1. See supplementary information for the determination of C/D from the raw intensity data. In a, b and c the dashed black vertical lines located at $t = 137$ s indicates the transition from the phase separation regime to the self-assembly regime, when the membrane of the vesicle start to form. d, schematic of the polymer structure used in the SCF computations and example concentration profile. e. Evolution of the polymer interfacial area, α , and aggregation number, g , as function of the solvent composition, expressed as water volume fraction, ϕ_W from the SCF simulations. f, Evolution of average end-to-end distance of the PCL, ϵ , and PEO, δ , blocks as a function of increasing polymer volume fraction in the membrane, ϕ_{PCL} , from the SCF simulations. g, Evolution of membrane thickness with contrast from the LPEM data, lines fits from raw data, in b and c. h, Schematic illustrating the molecular reorganization of the polymer as ϕ_W increases obtained by the SCF simulations.

Quantitative analysis of vesicle formation. To understand the polymer-rich liquid droplet to vesicle transformation we use the cross-sectional time series and radially averaged intensity profiles to measure changes in particle diameter (D , Fig. 5a), membrane thickness (T , Fig. 5b) and particle contrast (C , Fig. 5c) determined for 5 vesicles (see supplementary information). We separate the formation into two regimes, growth of the polymer-rich liquid droplet ($t < 137$ s) and nucleation and growth of the assemblies ($t > 137$ s). The contrast of the particles is related to their composition, and an increase in contrast is indicative of an increase in polymer concentration. To compare individual particles, we have normalized the contrast based on the particle diameter (see supplementary information). In the early stages ($t < 137$ s) the increase in size and contrast is related to the growth of the polymer-rich liquid droplet by accumulation of polymer from the bulk solution. In the later stages ($t > 137$ s) the size of the particles stabilizes as self-assembly becomes preferred over phase separation, and the liquid droplet begins to evolve into the vesicle. During this process the contrast at the interface between the droplet and the solution continues to increase. This indicates that after self-assembly becomes favoured, the vesicles continue to form via addition of polymer from the bulk solution (rather than simply a reorganization of polymer inside the liquid phase precursor). While the general trends are the same, each individual vesicle follows its own unique pathway of formation.

To gain a molecular understanding of the individual particle self-assembly pathways we compare the experimental results with the SCF simulations. The computations were performed on block copolymers with and without molar mass dispersity, to simulate the measured dispersity in our system and to understand its effect on the formation process (Supplementary Fig. 20-22). By labelling monomers at the end of the two blocks and the junction between the PCL and PEO blocks enables us in our computations, to track the end-to-end distance of each block, which is related to the change in thickness of the membrane and the stretching of the polymer chains (Fig. 5d). For a bilayer morphology, the interfacial area (α) decreases with increasing water volume fraction in the environment (ϕ_w) (Fig. 5e). Simultaneously, the volume fraction of PCL in the membrane (ϕ_{PCL})

increases, which is essentially a replacement of the solvent molecules inside the membrane for polymer molecules from solution. This results in increased steric repulsion between the PCL and PEO blocks in the membrane and consequently the blocks stretch, leading to an increase in membrane thickness. In fact, for the entire assembly process the SCF computations predict a linear relationship between ϕ_{PCL} and the average end-to-end distance of the PCL and PEO blocks (ε and δ , respectively), where ε represents half the membrane core thickness for a perfect bilayer (Fig. 5f). This linear relationship can be considered a hallmark of the equilibrium assembly process for this system. By comparing the change in contrast (related to ϕ_{PCL}) to the change in membrane thickness (related to ε) we can determine the thermodynamic and kinetic contributions to the assembly process for each vesicle (Fig. 5g). Vesicle 5 displays a near linear relationship between contrast and thickness for the entire movie, as predicted by SCF simulations. The other vesicles deviate from linearity at different stages and to different degrees, but all showing an increase in contrast without the predicted increase in thickness, indicating pathways in which kinetic traps occur. Intuitively, deviations from the thermodynamic pathway would be expected to occur at either a defined membrane thickness, or a defined water composition when the dynamics of reorganization becomes slow compared to the rate of mixing.¹⁷ However, the differences observed in Fig. 5g, indicate that the onset of the kinetic traps is specific to the organization or composition at the level of a single particle.

Conclusion

From our data we can see that the polymer-rich liquid droplets can affect the assembly process of our model system in several ways. Firstly, the assemblies form at the interface between the droplet and the bulk solution. This means that the nucleation and growth of the assemblies will be dictated by the size and composition of the droplet. Secondly, after the assemblies start to form, the size of the polymer-rich liquid droplet stabilises and determines the final size of the vesicles. Thirdly, the onset of kinetic traps plays a role in determining the membrane thickness, and is related to the specific organization or composition of the assemblies. As the liquid precursor controls the nucleation and growth of the assemblies it will also play a role in determining the thermodynamic and kinetic pathways of the assemblies. Since the nucleation of the precursor droplet is a stochastic process, the growth kinetics are likely defined by the experimental conditions, for example, polymer concentration, and the rate at which the solvent quality is varied. This could explain why the formation of disperse vesicles with no preferred curvature has often been observed experimentally,⁵⁵ and why mixing rate during self-assembly has been shown to influence vesicle size, which was previously ascribed to the formation of pure polymer domains.⁴⁸ Our data also indicates that the kinetics with which the thermodynamic regimes are traversed can effect both the details of the formation mechanism and the final morphology. Finally, although we are not studying encapsulation directly, Adams *et al.*⁵⁶ have shown that the encapsulation of hydrophilic molecules in the internal water pool of vesicles is dependent on the formation mechanism. Here, we propose that encapsulation can be controlled by creating models that consider the encapsulants affinity for the polymer-rich droplet as well as the thermodynamic and kinetic transport processes across the patchy membrane.

Schmid and He proposed a new vesicle formation mechanism based on external potential dynamics (EPD) simulations,⁵⁷ which has been supported by several other theoretical studies¹⁰. They proposed that vesicles form via the self-assembly of spherical micelles, which grow isotropically,

and at a critical size, internally reorganize to create the membrane⁵⁷, rather than first forming cylinders that transform into vesicles^{6,17}. Here, our model describes the spherical growth and internal phase separation of a polymer-rich liquid phase precursor, which evolves as the assemblies form at the interface with the solvent rich phase. These assemblies are initially spherical and then transform into cylinders or lamella before forming the vesicle membrane. It is interesting to note that morphological features of each previously proposed mechanism can be observed within a single vesicle, depending on how the information is displayed (Fig. 4a and b). Hence, our proposed model offers a unifying description of vesicle formation, whereby the previous pathways can be considered as two extreme differences in how the self-assembled structure evolve inside the previously unknown liquid phase precursor (see supplementary information for a more detailed description and Supplementary Fig. 23).²⁴ Considering our model predicts that liquid-liquid phase separation should occur in a wide range of amphiphilic systems, we anticipate their consideration during the design of amphiphilic assemblies will afford a greater control over the structure and properties of the resulting materials.

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Data availability The SCF input files, the data generated by the SCF computations, and the source data for the figures are available from the corresponding author upon reasonable request

Code Availability The matlab script used for image analysis are available from the corresponding author upon reasonable request. The SCF code has been provided by prof. Frans Leermakers of Wageningen university.

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Author contribution: J.P. and N.S. supervised the study; J.P., N.S. and A.I conceived the experiments; H.W. J.P., and P.V. performed the LP-TEM experiments; A.C.E. and A.I. were responsible for the polymer synthesis and characterization, R.T. and A.I. developed the SCF computations. A.I. developed the theoretical framework; H.F. supervised the movie analysis, A.K. removed the duplicate frames and stabilized the movies; H.W. developed and conducted the sub-alignments and quantitative analysis; M.v.R. performed the cryo-TEM experiments; J.P. wrote the

paper with contributions from all authors; all authors discussed the results and commented on the manuscript; A.I and H. W contributed equally to this work.

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Table of contents:

Although amphiphilic self-assembly is ubiquitous in science and nature, the underlying mechanisms governing the transition from molecules to assemblies have not been fully resolved. Here, liquid phase electron microscopy, self consistent field computations and Gibbs free energy calculations reveal the role that liquid-liquid phase separation plays during amphiphilic self-assembly.