Inward growth by nucleation: Multiscale self-assembly of ordered membranes

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Striking morphological similarities found between superstructures of a wide variety of seemingly unrelated crystalline membrane systems hint at the existence of a common formation mechanism. Resembling systems such as multiwalled carbon nanotubes, bacterial protein shells, or peptide nanotubes, the self-assembly of SDS/β-cyclodextrin complexes leads to monodisperse multilamellar microtubes. We uncover the mechanism of this hierarchical self-assembly process by time-resolved small- and ultrasmall-angle x-ray scattering. In particular, we show that symmetric crystalline bilayers bend into hollow cylinders as a consequence of membrane line tension and an anisotropic elastic modulus. Starting from single-walled microtubes, successive nucleation of new cylinders inside preexisting ones drives an inward growth. As both the driving forces that underlie the self-assembly behavior and the resulting morphologies are common to systems of ordered membranes, we believe that this formation mechanism has a similarly general applicability.

INTRODUCTION

The superstructures that occur in self-assemblies of crystalline membranes often share a set of common morphologies: They form hollow shells, single- or multiwalled (nano)tubes, or lamellae. Single- and multiwalled (nano)tubes are found in self-assemblies of amphiphilic peptides (1–3) and the allotropes of carbon (4–8). The same morphologies are also found in living systems such as microtubuli (9–11), bacterial protein shells (12), and bacterial chlorosomes (13–15). The striking morphological similarities between these seemingly unrelated systems suggest that the driving forces underlying their self-assembly mechanisms are common to many of these systems, depending more on the interplay between rigidity and the high energy cost of membrane edges than on the nanoscopic details of the individual systems. Moreover, these types of system often share a remarkable degree of monodispersity. Hence, this suggests the existence of a well-defined formation mechanism, common to a broad range of systems of crystalline membranes.

The self-assembly of sodium dodecyl sulfate (SDS) and β-cyclodextrin (β-CD) leads to superstructures that are representative of the class of crystalline membrane materials. In the work of Jiang et al. (16–18) and Yang et al. (19), the rich phase behavior of the SDS/β-CD system was explored, showing, among others, multiwalled microtubes, polygonal capsids, and lamellar phases, as shown in Fig. 1. A common feature of all these superstructures is a rigid crystalline bilayer membrane, with a well-ordered internal structure that is identical regardless of the higher-order organization.

In our previous work (20), we demonstrated the use of small- and ultrasmall-angle x-ray scattering (SAXS and USAXS, respectively) to determine the structure of SDS/β-CD microtubes in situ. From the scattering profiles, we were able to quantify microtube radii and inter- and intrabilayer periodicities from the scattering profiles at low, intermediate, and high q, respectively. By measuring the small-angle scattering profiles, we essentially have access to structural information on multiple length scales, opening the door to detailed mechanistic studies of self-assembly processes. Here, we follow the kinetics of SDS/β-CD structure formation, using time-resolved SAXS. From the resulting structural information, we uncover the mechanism of microtube self-assembly.

RESULTS

Kinetic measurements

We obtained USAXS patterns of temperature-quenched solutions of [SDS@2β-CD] in the process of self-assembly on the USAXS beamline ID02 at the European Synchrotron Radiation Facility (ESRF). The technique was previously used to follow the self-assembly kinetics of various systems, such as virus capsids (21, 22). In a typical experiment, a heated, premade solution of [SDS@2β-CD] was injected in an observation capillary kept at room temperature, effecting a rapid temperature jump. Immediately after injection, SAXS patterns were recorded in regular, increasing intervals. Figure 2 shows the azimuthally averaged SAXS profiles of a typical experiment as a function of time after injection. The initial SAXS intensities correspond to the form factor of the [SDS@2β-CD] complex in solution, which is formed as the hydrophobic tail of the SDS molecule is inserted into the hydrophobic pocket of two β-CD molecules. The data show an initial waiting time before the scattering profile visibly changes. After this initial waiting time, which we denote as t_w, there is a significant increase in scattering at small angles. Simultaneously, peaks appear at higher angles, corresponding to the in-plane organization of the [SDS@2β-CD] complexes. These peaks do not shift during the course of an experiment, a confirmation that the in-plane organization of [SDS@2β-CD] complexes is unchanged throughout the self-assembly process.

Initial formation of cylinders

During the increase in scattering intensity, oscillations are visible at small angles: Up to 20 orders can be observed during these intermediate stages of the self-assembly. The oscillations appear throughout our experiments, although they are clearest in samples when the [SDS@2β-CD] concentration is around 6 to 10 wt %. The oscillations only dampen out after a large number of orders. This indicates that the structure present in the sample is highly monodisperse, with a standard deviation (SD) in the average radius well below 5%. We plot a subset of the

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SAXS profiles of the experiment, with one curve highlighted showing pronounced oscillations, in Fig. 3. The scattering intensity follows an inverse square decay, the expected scaling for a two-dimensional (2D) object. Moreover, the profile closely resembles the theoretical form factor of hollow cylinders (23).

The intermediate structures observed in Fig. 3A are extremely monodisperse, which is surprising, because symmetrical bilayers are expected to have zero preferential curvature. It is likely that the edges of \([\text{SDS@2β-CD}]\) bilayers are highly unstable, an indication of which is given by the very low solubility of β-CD in comparison to other cyclodextrins (24). Hence, the energy cost of bending the bilayer into a cylindrical geometry is compensated by the large gain in bond free energy provided upon closure of the cylinder. Competition between bond formation and bending free energy leads to a minimum in the free energy per complex, which we show in fig. S3. A similar mechanism has been observed in the formation of monodisperse spherical vesicles from fluid membranes (25, 26). We provide arguments in the Supplementary Materials that support a preference for a cylindrical geometry in crystalline membranes.

**Microtube radius and inward growth**

We show the azimuthally integrated SAXS profiles of a typical experiment in Fig. 3C. The profiles were fitted to the form factor of long hollow cylinders with a central radius \((r)\) and a thickness \((\delta)\), given by the equation (23, 27)

\[
I(q) = \frac{2\pi^2}{q} \left[ \frac{(r + \frac{\delta}{2})J_1(q(r + \frac{\delta}{2}))}{q} - \frac{(r - \frac{\delta}{2})J_1(q(r - \frac{\delta}{2}))}{q} \right]^2 \tag{1}
\]

where \(l\) is the total length of the cylinders and \(J_1\) is the Bessel function of the first kind. While the equation is an approximation, we show in the Supplementary Materials that it provides good approximations for the mean radius and multilamellar stack thickness even when the cylinder length and radius are comparable. The resulting radii and thicknesses are plotted as a function of time in Fig. 3D. For comparison, we plot the Porod invariant \(Q = \int q^2 \times I(q) dq\) (28), a measure for the amount of ordered material present at each point in time during the self-assembly process, in Fig. 3E. The average central radius \((r)\) initially decreases rapidly, followed by a stabilization at longer time scales. Our fits show a concurrent increase in the cylinder thickness \((\delta)\), consistent with a constant outer radius equal to \(r_0 = (r) + (\delta)/2\). Hence, we attribute this effect to an inward growth process. If the growth is directed inward, then the
outer radius of the microtubes should remain constant throughout an experiment, which is confirmed by the observed positions of the scattering minima at low $q$ (~0.005 nm$^{-1}$) in Fig. 2 and in optical microscopy observations shown in fig. S1.

The experiment was repeated for a number of different [SDS@2β-CD] concentrations. The resulting evolutions of the average central radius ($r$) are plotted in Fig. 4A. All experiments show a similar decrease in average radius. The slight increase of average radius at the end of the experiment likely reflects a separate relaxation process that is caused by convective processes within the scattering cell.

The presence of a concentration-dependent initial waiting time is reminiscent of a nucleation process. Classical nucleation theory was introduced in the 1920s and 1930s (29–31) as a means to model the homogeneous nucleation rate of a supersaturated solution in microscopic terms. Within the framework, the nucleation rate depends on the concentration in a highly nonlinear way, depending on the dimensionality of a critical nucleus. For a disc-shaped 2D critical nucleus, the nucleation rate follows an Arrhenius-type equation, given by

$$j = A \exp \left( -\frac{\pi^2 a_0}{k_B T \Delta \mu} \right) = A \exp \left( -\frac{\pi^2 a_0}{(k_B T)^2 \log \frac{c}{c^*}} \right)$$

where $j$ is the nucleation rate within the observed volume, $\tau$ is the microscopic line tension of the edge of the disc, $a_0$ is the molecular surface area occupied by a single [SDS@2β-CD] complex, and $A$ is a kinetic prefactor. The chemical potential $\Delta \mu = -k_B T \log S$ is a function of the degree of supersaturation $S = c/c^*$, where $c$ is the concentration of [SDS@2β-CD] complexes and $c^*$ is the saturation concentration, which we estimate at 3 wt % (see Fig. 1). A derivation of the equation for a disc-shaped critical nucleus is presented in the Supplementary Materials. If we take the reciprocal of the nucleation rate as a typical nucleation time scale, $t_0 \sim j^{-1}$, then the theory predicts that the logarithm of the typical nucleation time scale should be inversely proportional to the logarithm of the degree of supersaturation, with an intercept determined by the preexponential factor $A$. We plot the logarithm of the initial waiting time, $\log t_0$, as a function of $\log \frac{c}{c^*}$ in Fig. 4B. The data are convincingly described by a linear function. From the extrapolated intercept, we determined the preexponential factor to be $A = 1.5$ s$^{-1}$. Moreover, if we rescale the time axis of the experiments in Fig. 4A and plot the evolution of the average central radius ($r$) as a function of $\log t A \times \log \frac{c}{c^*}$ in Fig. 4C, then the data from all experiments collapse onto a single curve: The full kinetics of inward growth show classical nucleation scaling with concentration.

The mechanism of microtube formation can therefore be understood as illustrated in Fig. 5. Complexes of [SDS@2β-CD] in a supersaturated solution (Fig. 5A) nucleate into critical (2D) nuclei (Fig. 5B),...
which grow into bilayers. When a bilayer reaches a size large enough that the bending free energy can be overcome, cylinder closure occurs (Fig. 5C). The minimum in free energy, arising from a competition between bending free energy and bond free energy, leads to a population of monodisperse, single-walled cylinders. Cylinder closure occurs while the process of nucleation continues, and critical nuclei are formed on both the inside and outside of existing cylinders (Fig. 5D). Sheets formed within the cylinders are confined and can only form cylinders that are slightly smaller than their preferred size (Fig. 5E). Successive nucleation on the inside of existing cylinders causes the formation of a stack of concentric cylinders. Nucleation of new cylinders on the outside of existing cylinders occurs until the volume is closely packed (Fig. 5F). It is likely that the outer layers are deformed due to packing effects, causing the decay of the higher-order oscillations that are so apparent in the intermediate stages of the self-assembly process (27).

Within this mechanism, the growth of individual bilayers is constrained. Bonds with neighboring complexes occur only within the plane of the bilayer, and consequently, growth in thickness only occurs by nucleation of a new bilayer to form a stack. The width of a bilayer grows until cylinder closure occurs, and additional growth also has to occur by nucleation of new bilayers. In length, growth is constrained by the presence of other microtubes. Macroscopic growth can therefore only occur in width or in thickness, both exhibiting nucleation kinetics.

Hence, the average microtube thickness should scale with the square root of the total amount of bilayers. We plot the average cylinder thickness \( \langle d \rangle \), calculated from the shift in \( \langle r \rangle \), as a function of the Porod invariant \( Q \) over the later part of the experiment. The shaded areas in (A) and (C) denote the extent of the 95% confidence intervals.

At the start of the experiment, the growth in thickness is faster than the typical square root scaling, most likely due to the presence of nucleated bilayers in which cylinder closure has not occurred yet. These bilayers do contribute to the invariant \( Q \). While we have no structural probe to detect flat membranes in the same experiment, there is evidence that the [SDS@2β-CD] bilayers go through a flat intermediate stage: Yang et al. (19) observed thin, diamond-shaped sheets with an obtuse angle of 104°, identical to the obtuse angle of the rhombic bilayer lattice, as a minority product in self-assembled microtubes of [SDS@2β-CD] complexes. We speculate that a small fraction of bilayers has insufficient space available for cylinder closure, which can then be observed in the final structure by microscopy.

**Interbilayer separation**

Up to now, we did not consider the magnitude of the inward growth process. This magnitude is related to the typical spacing between successive concentric tubes. We traced the average spacing between...
Growing bilayer Cylinder closure energy. (Bonding with their neighbors. When the bilayer reaches a certain size, it becomes advantageous to close the ring, gaining bond free energy at the cost of bending free energy. Because nucleation and growth are not separated, new bilayers keep nucleating, both inside and outside preexisting tubes. Bilayers that nucleated outside preexisting tubes form new tubes. Bilayers that nucleated inside preexisting tubes are restricted in their size and form concentric inner cylinders. Evidence for this deformation is given in fig. S2.

We put forward a model to explain the observed concentration scaling based on Helfrich’s expression for the bending free energy of membranes. We derived an expression for the average bending free energy per unit interface \( f \) bend for a set of concentric cylinders, which to a first-order approximation reads

\[
 f = \frac{\kappa}{2r_0^2} + \frac{\kappa}{2r_0^2} \sqrt{5c_0d} + \ldots
\]

where \( \kappa \) is the mean elastic modulus of the membrane, \( r_0 \) is the radius of the outermost cylinder, \( a_0 \) is the interfacial area occupied by a single \([SDS@2\beta]-CD\) complex, \( c \) is the number density of \([SDS@2\beta]-CD\) complexes, and \( d \) is the interbilayer separation. A full derivation of this expression is given in the Supplementary Materials. For interbilayer separations comparable to the Debye length, the weak overlap Debye-Hückel approximation is not very accurate. Instead, we use the expression for disjoining pressure due to electrical double-layer repulsion, derived by Philipse et al. \((36, 37)\) for a system of parallel plates with extensive double-layer overlap. The electrical free energy per unit interface is given within this framework as

\[
 f_{el} = \frac{k_B T \sigma^2}{d} \frac{1}{\rho_s}
\]

where \( \sigma \) is the surface charge number density of the membrane and \( \rho_s \) is the salt number density. The interbilayer spacing that optimizes the total free energy is then found by taking the partial derivative of \( f \) with multiple bilayers from the periodicity of the peaks present at intermediate angles. We show azimuthally integrated SAXS profiles of a typical experiment, multiplied by \( q^2 \) to decouple the bilayer structure factor from the cylinder form factor with apparent \( q^{-2} \) decay, in Fig. 6A. The maxima of the peaks were consequently found by cubic interpolation and are shown superimposed on the scattering profiles in Fig. 6A. As the amount of ordered material increases, the average spacing \( \langle d \rangle \) increases slightly over time, as we show in Fig. 6B and C.

The interbilayer separation can be understood as a competition between electrical double-layer repulsion between charged interfaces and the increased energy cost of bending the \([SDS@2\beta]-CD\) membranes into tighter cylinders. The electrical double-layer repulsion is affected by the salt in the system; however, addition of up to 10 M NaCl has no measurable impact on the interbilayer separation, as shown in the Supplementary Materials. Likely, a fraction of \([SDS@2\beta]-CD\) complexes is not incorporated in bilayers but acts as an additional salt reservoir. The increase in interbilayer separation visible in Fig. 6B is then likely caused by lower screening, as more \([SDS@2\beta]-CD\) complexes are incorporated in bilayers.

The final interbilayer separation is concentration-dependent. We measured the SAXS patterns of a concentration series of preassembled \([SDS@2\beta]-CD\) samples on the Dutch-Belgian Beamline (DUBBLE) at the ESRF, as shown in Fig. 6D. We determined the average interbilayer separation from the average peak separation, which we plot as a function of concentration in Fig. 6E. For samples of a concentration above 25 wt %, the interbilayer separation scales with the reciprocal of the concentration, expected for a purely lamellar phase. In the concentration regime where microtubes occur, the scaling is sublinear, reflecting the more compacted structure with open central pores in each microtube.
respect to \( d \) and setting it to zero. We found that the optimal interbilayer spacing \( d^* \) is given by

\[
d^* = \left( \frac{k_B T \sigma^2}{\rho_s} \right)^{1/2} \frac{4 \pi \sigma_0^2}{\sqrt{3} \Delta_0 k_C} \propto \frac{1}{\sqrt{c}}
\]

The predicted \( 1/\sqrt{c} \) scaling matches convincingly with the experimentally observed concentration dependence. There are three free parameters: \( \kappa, \sigma, \) and \( \rho_s \). We estimate the bulk salt concentration \( \rho_s \) to be approximately \( 10^{-3} \text{M} \) from the results of the experiment shown in fig. S5. The mean elastic modulus \( \kappa \) and surface charge density \( \sigma \) cannot be determined independently from this experiment. Double-layer lipid membranes typically have mean elastic moduli on the order of \( 10^2 k_B T \), and we estimate that the [SDS@2\( \beta \)-CD] bilayers are somewhat stiffer due to their high degree of order. If we estimate \( \kappa \) to be on the order of \( 100 k_B T \), then we find from the resulting surface charge density that a large fraction of counterions are condensed, leaving approximately 0.15% of all [SDS@2\( \beta \)-CD] complexes in the bilayers charged. While the weak-field approach of Philipse et al. (36) was shown to match accurately with numerical solutions of the Poisson-Boltzmann equation, to our knowledge, this is the first quantitative demonstration of the predicted \( 1/d \) scaling in an experimental system.

**DISCUSSION**

We measured the kinetics of the hierarchical self-assembly of SDS and \( \beta \)-CD into multiwalled microtubes using time-resolved (U)SAXS after a rapid temperature quench. The [SDS@2\( \beta \)-CD] complexes in solution were found to nucleate into highly ordered, rhombic bilayers that close into cylinders when reaching a sufficiently large size. The cylindrical geometry is likely an effect of an anisotropic elastic modulus, as indicated by the correlation between the alignment of the planar, rhombic [SDS@2\( \beta \)-CD] lattice and the overall microtube direction.

The nucleation process follows the kinetics of classical nucleation. The observed inward growth of the microtubes is caused by successive nucleation of new [SDS@2\( \beta \)-CD] bilayers inside existing cylinders, until a densely packed system of concentric cylinders is obtained. The interbilayer separation was found to be determined by a competition between electrical double-layer repulsion forces and the (increased) energy penalty of bending the [SDS@2\( \beta \)-CD] membranes into tighter cylinders. We found that the optimal interbilayer separation scales with the inverse square root of the concentration, which is confirmed by static SAXS experiments on preassembled systems of [SDS@2\( \beta \)-CD] microtubes.

In general, we argue that the driving forces that cause the formation of the elaborate microtubes do not depend on the specific chemistry of the molecular species involved. Instead, the mesoscopic behavior is
direct consequence of three “ingredients”: the (anisotropic) bending rigidity of ordered membranes, a repulsion between the faces of the membranes, and an interfacial tension at the edges of the membrane. It is likely that the mechanism presented here has a far greater applicability than within this particular system, for example, in the behavior of biological crystalline membranes, where all three ingredients are present. In the case of carbon allotropes, the dominant repulsive contribution is of much shorter range, and the interlayer separation is determined more by the discrete nature of the lattice. However, even with that caveat, the overall morphology is strongly influenced by the bending rigidity and the high energy of dangling edge bonds, and as such, we believe that a similar mechanism may be applicable to the nucleation of carbon nanotubes in the absence of a heterogeneous catalyst.

MATERIALS AND METHODS

Solutions of [SDS@β-CD] inclusion complexes

Desired amounts of SDS (>99%; Sigma-Aldrich) and β-CD (97%; Sigma-Aldrich) were weighed and mixed with ultrapure water (Milli-Q), keeping a constant β-CD to SDS ratio of 2:1. The samples were heated to 75°C, yielding clear solutions. The samples were allowed to cool down to room temperature, resulting in a turbid and viscous suspension.

Confocal laser scanning microscopy

Samples were dyed by mixing 10 μl of a solution of Nile red (1 mg ml⁻¹) (>98%; Sigma-Aldrich) in acetone with a sample at 60°C and allowing the acetone to evaporate. Observation samples were prepared by sandwiching a droplet of dyed suspension between glass coverslides (0.17 mm; Menzel Gläser). Images were recorded on a Nikon TE2000U inverted microscope with C1 confocal scan head and a 100× Nikon oil objective, with a 543.5-nm HeNe laser (Melles Griot) as an excitation source.

Small-angle x-ray scattering

SAXS patterns were recorded at the high-brilliance beamline ID02 (kinetic measurements) and the DUBBLE BM-26B (concentration series) at the ESRF (38, 39). For the kinetic measurements, we used two different sample-to-detector distances to cover the q range needed in our experiments. Data were recorded using a Rayonix MX170-HS detector, placed at 30.7 m for the smallest angles and at 2.5 m for the larger angles. The setup allowed measurement of scattering profiles from q = 0.0015 to 5 nm⁻¹, where q = 4π sin θ/λ where θ is the angle between incident and scattered x-rays. For the concentration series, scattering data were recorded using a Pilatus 1M detector at 1.5 m. For all measurements, background corrections were performed on all radial intensity profiles. A background measurement of pure water was obtained before each experiment. In all experiments, the x-ray wavelength was λ = 0.1 nm.

Kinetic measurements

A BioLogic SFM400 Stopped-Flow mixing device was used in the kinetic measurements to effect a fast (~ms) temperature jump. The SFM400 was equipped with four syringes in the main reservoir. The setup and data acquisition scheme are described in more detail by Narayanan et al. (40). Heated samples (75°C) were loaded in one of the syringes, and the others were filled with ultrapure water. The reservoir was kept at 60° or 75°C (for 20 wt % samples). The syringes and mixers were coupled to a thermostated quartz 1.5-mm-diameter observation capillary, kept at room temperature, and placed in the path of the x-ray beam at the ID02 beamline. In a typical experiment, the observation capillary was flushed with ultrapure water and a background measurement was obtained. The triggered release of 200 μl of sample into the observation capillary was followed by the time-resolved recording of the SAXS patterns. Time between measurements started at 8 ms and was increased by a factor of 1.02 for each measurement. A hard stop in the outlet of the capillary was activated before the first measurement to prevent continuous flow and to dampen any remnant flows from the injection. The observation volume is sufficiently small that the temperature quench occurs in a matter of milliseconds. This was verified by the absolute scattering level of water in the compressibility limit, which is sensitive to changes in the temperature. Within the time resolution of the setup, no signature of an elevated temperature was detected after the injection of hot water. Assuming that the heat transfer and thermal capacity of the SDS/β-CD solution are of the same order of magnitude, the temperature jump should be similarly fast.

The waiting time t₀ was defined as the point where the Porod invariant Q visibly starts to increase. To prevent bias, we estimated the waiting times from a number of different experimental sets before plotting in Fig. 4B.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/6/eaat1817/DC1

Supplementary Text

fig. S1. Optical microscopy during self-assembly.

fig. S2. Melting of microtubes follows reverse process.

fig. S3. Competition between bond formation and bending energy.

fig. S4. Chiral pitch of cylinders.

fig. S5. Influence of salt on the interbilayer separation.

fig. S6. Form factor fitting.

Reference (41)

REFERENCES AND NOTES


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