Rapid Spontaneous Assembly of Single Component Liposomes

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Can single component bilayer forming surfactants spontaneously assemble to form unilamellar vesicles of a definite size? Although possible by Helfrich’s theory, this has not been observed without external forcing. We show two variants of a method where this is possible without any input of external energy, with the time of synthesis reduced to as less as 15 minutes. It is shown that the average diameter of the liposomes formed is intrinsic and depends only on the temperature and the lipid type, eliminating kinetic effects normally observed. This thus can be a candidate mechanism for vesicle size selection in pre-biotic conditions. The method may also be suitable to study time-resolved studies of micelle to vesicle transition.

Lipid vesicles (or liposomes) are thought to play an important role in the development of cellular life forms [1] and are also used in formulations for targeted drug delivery [2]. Laboratory methods to produce unilamellar vesicles of a definite size either rely on an external energy input (such as sonication or extrusion), using multi-component systems (such as catanionic, lecithin+bile, etc.) [8–12], or by inducing a spontaneous curvature through certain molecules [7, 13]. We hypothesize a mechanism of spontaneous assembly of a single component lipid from a solution phase which results in predictable diameter vesicles, and demonstrate it in a commonly used lipid system. This mechanism, which we call stationary phase interdiffusion (SPI), is applicable to any vesicle forming system, with suitably chosen solvents.

Uni-lamellar vesicles are meta-stable structures kinetically trapped in high energy states. The Gibbs energy (originating predominantly from the bending elasticity) of closed vesicle does not depend on its diameter, favouring coalescence of small vesicles to form larger vesicles (reducing the total energy by the count), although the transition barrier for this can be large (typically 20 times the thermal energy $kT$) [13]. The kinetic nature of the vesicular states is unlike other surfactant aggregates such as micelles which are in a dynamic equilibrium with the dilute (solution) phase. The vesicle diameter is therefore usually determined by external parameters from the sample preparation process which leads to one of these energy traps.

Nevertheless, there is one characteristic diameter, for vesicles assembled from a solution phase of lipids, obtained from a energy balance about a disc micelle aggregate (bilayer disc membrane) [15–17]. This diameter $D$ is determined only by a balance of hydrophobic edge energy of the disc and the elastic bending energy of the membrane, and is given by

\[
D \approx \frac{8(2\kappa + \bar{\kappa})}{\gamma} \tag{1}
\]

where $\kappa$ is the elastic bending modulus of the membrane, $\bar{\kappa}$ is the Gaussian curvature modulus, and $\gamma$ is the line (edge) tension of the membrane with the surrounding solvent. This diameter is also known as the “minimum” diameter, obtainable by intense sonication [15]. However, we choose to call it as an intrinsic size of the vesicle, for reasons that will be elaborated later, as $\kappa$, $\bar{\kappa}$, and $\gamma$ are thermodynamic parameters of the vesicle forming amphiphile in the surrounding solvent. For phototidylycholine lipid vesicles in water this diameter is about 10–30 nm (see Supplementary Material [C] for an estimation) [18–20]. However, this diameter, although being the only natural one, has not been observed by a spontaneous process for a single component lipid system (for multi-component systems, spontaneous formation occurs when kinetic effects of other surfactants plays a role in manipulating the edge energy [11]).

Strong hydrophobic interaction of double tailed surfactants and the high energy state of the intrinsic diameter are the chief reasons behind the difficulty of observing such a spontaneous assembly [21, 22]. The small critical aggregation concentrations of the phospholipids (typically less than a nM), does not permit efficient dispersion and mixing of the surfactants. The intrinsic diameter has, thus far, not been obtained by a spontaneous process but by dissipating energy into the system (by sonication or extrusion) [23].

We conceptualise a mechanism to observe the spontaneous assembly. Consider a uniformly dispersed solution of bilayer forming molecules. If the bulk (good) solvent is suddenly replaced by an aqueous (poor) solvent, the changed conditions favour aggregation of the molecules to form circular discs (bilayer membranes or bicelles). The system then gets trapped in its intrinsic size as the discs grow. Such a situation of “magically” replacing the surroundings can be easily achieved in computer simulations of molecules [24, 25], but the challenge is also to realise it in real experiments.

Instead of a bulk replacement, the solvents can be diffusively replaced across an interface, provided they are miscible. When the interdiffusion coefficient of the solvents is much large compared to that of the amphiphile molecule in the original solvent, these molecules are localised in space while the original solvent is replaced by the aqueous solvent by interdiffusion, as illustrated in the schematic shown in Figure [1]. This results in a condition analogous to the bulk replacement discussed above, albeit in a nearly two-dimensional region. This region of the solution is essentially subjected to a “quench” from a dilute or “gaseous” phase (of the amphiphiles) to a spinodal region leading to a bulk phase separation to the bilayer phase. The size would be intrinsic, but at a value determined by the thermodynamics of the lipid in the mixture of solvents.

To achieve this mode of assembly, three components are
essential: bilayer forming amphiphile (lipid or a block copolymer), a good solvent and a poor solvent which are intermiscible. We found a classical system—phosphotidylcholine lipid, ethanol, and water—well suited to capture the phenomenon described. Here, the lipids dissolved in ethanol is brought into contact with water across a stationary interface.

Owing to the high miscibility of water and ethanol the interfaces need to be brought into contact in a nearly stationary manner, taking care to avoid any convective (advection) mixing, that can influence the energetics of self-assembly [26] (see Supplementary Material A for a description of the setup and the method). Achieving the stationary contact sets this apart from the other methods using the same components [26-28]. As the interdiffusion proceeds, a turbid front is seen to move upwards, indicating the formation of liposomes (see Supplementary Material B for a detailed description and a video).

The liposome suspension obtained shows a monodisperse population with a mean hydrodynamic diameter equal to 530±25 nm for the dimyristoyl phosphotidyl choline (DMPC) lipid as seen in Figure 2(a). High resolution transmission electron microscope (HRTEM) images show that the liposomes are unilamellar for various types of lipids used (Figure 2(b-d)). An independent estimate of the intrinsic diameter using Equation C1 can be obtained. In the presence of ethanol we find this to be about 600 nm for the DMPC liposome, which is a good order of magnitude estimate of the experimentally observed hydrodynamic diameter of 530 nm (see Supplementary Material C for the calculations).

That the diameter is intrinsic is reinforced by two crucial observations. (i) The concentration of lipids does not have an influence on the diameter of vesicles as seen in Figure 3a. Higher concentration only leads to higher number density of vesicles of the same average size, as evidenced by the same number counts from scattering measurements achieved at a higher dilution and visually by a more turbid suspension (see Supplementary Material E for calculations supporting this claim). (ii) the proportion of ethanol and water on either side of the interface does not influence the size so long as the vesicles are formed, as seen in Figure 3b. We infer from this that the lipid assembly happens when the bulk concentration of ethanol in the interfacial region is between 25 and 50% (v/v).

The intrinsic size is expected to change when the moduli $\kappa$, $\bar{\kappa}$ or the edge tension $\gamma$ is altered. The temperature and lipid chemistry (chain length and unsaturation) are two handles that can readily affect a change in the diameter by this alteration. Liposomes are formed only at temperatures higher than the main transition temperature of the phospholipid ($T_m = 24^\circ C$ for DMPC), and has a significant effect on the size of liposomes as seen in Figure 4a. In the temperature range studied, the size of the DMPC liposomes shows an increase with temperature. In the temperature range 30–40$^\circ C$ the bending modulus is nearly constant at $\kappa \approx 1.3 \pm 0.1 \times 10^{-19}$ J [29]. However, it is expected that the edge tension decreases due to a known decrease in the interfacial tension as the temperature increases. Lipids of various chain lengths and unsaturation also form mono-disperse, unilamellar vesicles as seen in Figure 4b. We find that lipids with shorter alkyl chain length and those with larger number of unsaturated bonds form larger liposomes, as shown in Figure 4b, in agreement with an expected reduction in the bilayer thickness and hence the edge tension. Accurate measurements of the edge tension values and the bending modulus for different lipids in the presence of ethanol is required to further validate our claim.

We have avoided “minimum” and chose to introduce “intrinsic” diameter instead, for the following reasons: “Minimum” is appropriate only if we consider a system of lipids in a pure aqueous phase. In the water-ethanol system, we may say that the diameter (around 600 nm) is the minimum in the environment where the liposomes form (because the surrounding ethanol reduces the edge tension (see Supplementary Material C). But if the overall aqueous phase is in far excess of the ethanol phase, then after complete mixing of the water and ethanol phases, the liposomes will eventually be dispersed in this predominantly aqueous medium. Here, then the minimum diameter would be close to 20 nm (see Supplementary Material C). But the liposomes will continue to be in the higher size (600 nm, which has a lower Gibbs energy), which is no longer the minimum diameter! The diameter, dictated at the time of formation, is observed to be intrinsic to the system of water-solvent-lipid.

A remark about the time of synthesis is now in order. The entire process in the cuvette cell configuration takes about 36 hours. However, we have confirmed by DLS measurements that the liposome formation is nearly complete as soon as the turbid front reaches the top of the syringe section at about 6 hours (see Supplementary Material B). This is done by terminating the stationary contact of the syringe and mixing the phases mechanically. This itself is a significant reduction in the time of synthesis compared to other commonly used methods that require multiple steps to achieve a mono-dispersed
FIG. 2: Liposome characterisation (a) Monodisperse size distribution of DMPC liposomes as intensity of scattered light obtained by dynamic light scattering (DLS), shown in comparison with polystyrene standards of monodisperse population. Here, the average size of liposomes, determined from the intensity distribution, is 530±25 nm. (b-d) Negative staining micrographs from HRTEM showing a large unilamellar liposomes from various lipids.

FIG. 3: Liposome size is intrinsic (a) There is no effect of the lipid (DMPC) concentration initially dissolved in ethanol on the diameter of the liposomes formed. (b) Evidence that shows that concentration of ethanol or its gradients across the interface does not influence the diameter of liposomes. The points on the left (squares in blue) were obtained by the pre-mixing ethanol to the aqueous phase in the cuvette, whereas those on the right (circles in red) were obtained by pre-mixing water in the lipid+ethanol solution in the syringe tube. Except for a small region in ethanol fraction (between 25–50% v/v), where no liposomes are stably formed, the diameter of the liposomes is nearly constant at 510±50 nm.

A similar stationary contacting of phases can be achieved in a horizontal glass capillary, where the buoyancy driven mixing of the two phases is made negligible. This drastically reduces the liposome synthesis time to about 45 minutes (see Supplementary Material [12] without any intervention. This time can be further reduced to about 15 minutes by gently tapping the capillary, to release vapour bubbles that occlude the motion of the turbid front. This makes it the fastest known methods to synthesize unilamellar mono-disperse vesicles, starting from pure components. We believe the reason for the faster synthesis in a capillary setup is due to a phoretic motion of the liposomes driven by a concentration gradient of ethanol in water (diffusiophoresis, [30]) or because of a viscosity gradient, which in turn leads to faster mixing of water in ethanol in the opposite direction, furthering the assembly of liposomes.

We conclude the observed spontaneous assembly resulting in unilamellar liposomes is quantitatively well explained by Helfrich’s model [15]. Since this does not involve any external energy input, it can be considered as a possible mechanism to explain the formation of vesicles of a uniform size under prebiotic conditions—i.e., we now have physical mechanism to which a natural length scale can be attributed. Suitable molecular candidates (other than the chemically evolved phospholipid systems considered here) need to be identified [31].

Due to the stationarity of the SPI method it may now be possible to infer the dynamics the micelle to vesicle transition
in a single component system using time-resolved scattering, which has been studied only for mixed surfactant systems [32, 33]. Preliminary investigations reveal that using the SPI process it is also possible to achieve a high degree of encapsulation (more than 80%) of hydrophilic molecules (or drugs) by dissolving it in the ethanol phase.

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FIG. 4: Temperature and lipid type influences the diameter (a) Diameter of DMPC liposomes shows an increase with increase in temperature. (b) Liposome size also depends on the lipid type, at 27.5°C. Shown are four lipids with various values of acyl chain length:unsaturation: DMPC—14:0, DPPC—16:0, Soy PC—mixture-16:0 (14.9%), 18:0 (3.7%), 18:1 (11.4%), 18:2 (63%), 18:3 (5.7%) and DOPC—18:1.

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Supplementary Material A: Materials and Methods

a. Preparation of phospholipid solution. The phospholipids used to synthesize the liposomes are: 1,2-dimyristoyl-sn-glycero-3-phosphocholine (14:0-DMPC); 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (16:0-DPPC); and L-α-phosphatidylcholine (Soy PC, a mixture of various lipids: 18:2, 16:0, 18:1, 18:3, 18:0 phosphatidylcholine), all purchased from Avanti Polar Lipids, was used without further purification. 1,2-dioleoyl-sn-glycero-3-phosphocholine (18:1-DOPC) was purchased from Sigma Aldrich. Ethanol (AR grade) was purchased from Merck and MilliQ water was used for the aqueous medium. All the lipids from Avanti Polar Lipids were in solution form in chloroform (20 mg/ml). An appropriate volume of lipid solution is taken out in a round bottom flask (RBF) and the chloroform is evaporated from the lipid solution using a rotary evaporator. This forms a thin film on the glass surface of the RBF. The RBF is then kept in a vacuum desiccator overnight to remove all the traces of chloroform. An appropriate volume of ethanol is added to the RBF to make various concentrations of phospholipid in ethanol (5, 10, 20, 40, 80 mg/ml).

b. Device setup. The experimental setup is shown in Figure 5. The key components of the device are: a container for aqueous phase (square cuvette 10×40 mm) and organic phase container (cut syringe tube 4.8 mm inner diameter). As shown in the setup, a hole is made in the cuvette cap and the cut tube is inserted through it. The phospholipid solution in ethanol is filled in the tube (held by the vacuum of the syringe piston). The aqueous phase is then gently filled in the cuvette through a syringe needle pierced through the cuvette cap. The two phases—water and ethanol—are held in stable contact. The mixing occurs solely due to molecular diffusion of one phase into the other. Adequate care is taken not to introduce convective mixing in the two phases near the interface during the process.

c. Dynamic light scattering (DLS)/Photon correlation spectroscopy (PCS). The liposomes were characterized by DLS or PCS (Zetasizer nano ZS, Malvern, UK) for size and size distribution (polydispersity), without any further processing, except aqueous dilution, when required, for reliable measurements. The laser used internally is He-Ne laser with 633 nm wavelength and 4 mW power and the scattered light is detected at a single angle of 173°. The size and size distribution was calculated by a software (DTS 6.1, supplied by Malvern). The polydispersity and average diameter were evaluated using mono-modal or cumulant analysis method and the intensity-weighted-diameter of diameter was evaluated using multimodal/distribution analysis. Usual care is taken to prevent dust and other contaminants in the system.

d. TEM using Negative staining. High resolution transmission electron microscopy (Jeol, JEM-2100F) was used to visualize the liposome lamellarity. Negative staining method was used to observe the liposomes under the HR-TEM. An electron dense material—phosphotungstic acid (PTA) 2%w/v—is used as the negative staining agent. PTA is acidic in nature and needs to be neutralized; the pH is adjusted by a dilute NaOH solution. A drop of liposome solution (10 μl) is placed carefully on a carbon coated TEM grid with the help of a micropipette and kept for air drying for 10 minutes, which is followed by a placing drop of PTA. The sample was air dried before observing. The operating voltage is kept at 120 kV.

Supplementary Material B: Stages of Liposome formation in SPI

The phospholipid dissolved in ethanol is taken in an inverted tube and is in contact with an aqueous phase (cuvette) as shown in Figure 5. As soon as the interface contact is established, the progress of liposome formation is clearly visible in the form of a turbid front that moves upward through the ethanol section. Time lapse photographs of the visual changes observed are shown in Figure 6.

A copy of the time-lapse video of the formation of liposome suspension is available from:

http://goo.gl/KOW3VN

The video has been made from photographs taken in 10 min time intervals (the timestamp is labelled on the top of the cuvette, in the format DD:HH:MM). Three distinct phenomena are observed—(a) Initially, both the lipid and aqueous phases are clear, and soon after, a turbid layer is observed inside the inverted tube which moves upwards, indicating that liposomes are being formed. This is completed by about 6 hours (b) As the white region moves vertically, some amount of liposome suspension also comes out in the aqueous phase (cuvette) and goes up towards the water-air interface, due to the lower density of ethanol that is diffusing into the aqueous phase. This is followed by a downward movement of “strings” of suspension towards the bottom of the cuvette (at a steady velocity of about 1 μm per sec). (c) Finally the strings diffuse out to form a uniformly turbid milky white suspension.
FIG. 5: **Experimental setup** Stationary phase inter-diffusion apparatus comprises two components—a container for aqueous phase (cuvette) and a cut syringe tube containing phospholipid-ethanol phase held by the syringe piston. The needle is used to gently add the aqueous phase to generate a stationary contact with the ethanol phase.

![Experimental setup diagram](image)

FIG. 6: **Stages of liposome formation** Time-lapse photographs of liposome formation; each photograph is taken in a time interval of 5 hours, showing interdiffusion and turbidity of liposome formation. After the initial contact, a turbid front moves upward in the ethanol medium, indicating liposome formation as water diffuses in. At around 6 hours the entire section of the phase inside the cut-syringe becomes uniformly turbid. After about 15 hours, strings of suspension move downwards, which laterally diffuse to form a uniform suspension by about 36 hours. The time stamp is labelled at the top of each frame in the format DD:MM:HH (denoting day, minute, and hour respectively).

The time of 6 hours for the first stage roughly agrees with the time scale of diffusion of water through ethanol through a distance of 1 cm, the length of the ethanol section in the cut-syringe. In the second stage, we believe, the strings form because of a viscous fingering instability occurring due to a mean motion generated by diffusiophoresis of the liposomes in a gradient of ethanol. Further experiments and analyses are underway to confirm this claim.

**Supplementary Material C: Estimate of the Intrinsic Diameter**

The intrinsic diameter is given by [15–17]:

\[
D \approx 8 \frac{(2\kappa + \bar{\kappa})}{\gamma}.
\]  

While \( \kappa \) is measurable easily, the Gaussian curvature modulus \( \bar{\kappa} \) has not be extensively measured, and is often taken to be equal to \( \kappa \). Recent coarse-grained simulation of lipids [34] have shown that the equalisation is indeed correct to within 10% accuracy. Since we are only providing an estimate of the
diameter we can comfortably write the diameter as

\[ D \approx 24 \frac{\kappa}{\gamma} \]  \hspace{1cm} (C2)

In the SPI method, the hydrodynamic diameter of the liposomes was found to be 530 nm for the DMPC lipids. Our claim that this is the intrinsic diameter can be validated by using independent measurements. For DMPC bilayer in water [29] with \( \kappa = 1.5\pm0.06 \times 10^{-19} \text{ J} \) and \( \gamma \approx 140 \text{ pN} \) (using water-oil interfacial tension of 35 mN/m and a bilayer thickness of 4 nm) gives a theoretical diameter of 25 nm. Experimentally, it is observed that more than two hours of sonication results in a diameter of 17 \pm 6 nm [23]. In the present case with the self-assembly taking place in the presence of ethanol, the estimates will differ. The bending modulus of lipid membranes is reduced in the presence of ethanol by about 30% at 20% v/v of ethanol for SOPC membrane vesicles [18]. This implies for DMPC we can expect \( \kappa \approx 1 \times 10^{-19} \text{ J} \) in the presence of ethanol. More significant is the reduction in the line tension alcohol can induce to an oil water interface. We estimate it to be \( \gamma \approx 4 \text{ pN} \) (using an interfacial tension \( \approx 1 \text{ mN/m} \) [19] and a bilayer thickness of 4 nm). This gives an estimated vesicle diameter of 600 nm, which agrees to within an order of magnitude of the experimentally observed value of 530 nm, and is reasonable given approximate nature of the property estimation we have used. More accurate measurements of the either the interfacial or the edge tension [20] for various lipids in the presence of ethanol may confirm our hypothesis of the intrinsic size.

**Supplementary Material D: Capillary Setup for Stationary Phase Inter-diffusion**

The cuvette and syringe method (Figure 5) takes \( \sim 8 \) hours to form the liposomes (1 cm height and 4.8 mm diameter tube). The formation time is observed to be considerably slower when capillaries are used. Glass capillaries 10 cm long and 1 mm in inner diameter is used with one end sealed. The procedure in short is—the aqueous phase is filled up to 8 cm with the help of a glass syringe attached to a 12 cm long 30 G SS needle; The phospholipid-ethanol solution is taken in another syringe and filled up to 1.5 cm by keeping a 1/2 cm air gap between the phases. Then the two miscible solutions are brought in contact by sucking out the air bubble through another needle. Care is taken to avoid any convective mixing at the interface otherwise a multi-modal size distribution of liposomes is observed. The total time required to form liposomes is less than an 45 minutes as seen in Figure 7 making it the fastest known way to synthesise mono-disperse liposomes.

Here again, we believe that the reason behind the fast movement of the turbid suspension is due to diffusiophoresis.

**Supplementary Material E: Concentration of Liposome Suspension**

As shown in the main communication, in the SPI process the concentration of lipids does not influence the size of liposomes. Increasing the concentration of lipids only results in more number of liposomes formed. We observe a suspension of a very high liposome fraction, as visually seen by a highly turbid mass inside the cut-syringe section. However, we were unable to exactly quantify the concentration; as shown in Figure 6, a part of the liposome suspension comes out of the cut syringe section as the turbid front moves upwards. This makes it difficult obtain accurate measurements. However, an estimate of the volume fraction of the liposome suspension can be obtained based on the average liposome diameter and the amount of lipids used.

Assuming that all of the lipids dissolved in ethanol form liposomes of the same size, and all of them are contained in the cut-syringe section, we can estimate the volume fraction of the liposome suspension in the cut-syringe section:

\[ \phi = \frac{c N_A D a_0}{12 M} \]  \hspace{1cm} (E1)

Here, \( c \) is the mass concentration of lipids, \( N_A \) is the Avogadro number, \( D \) is the average diameter of the liposomes, \( a_0 \) is the area per head group of lipid, and \( M \) is the molecular weight of lipid.

For DMPC lipid, \( M = 678 \text{ g/mol}, \) and \( a_0 = 0.71 \text{ nm}^2 \). From the DLS measurements we obtain the diameter of liposomes \( D \approx 500 \text{ nm} \). For a concentration of lipids at \( c = 20 \text{ mg/ml} \), we obtain the volume fraction to be

\[ \phi \approx 0.5 \]  \hspace{1cm} (E2)

which is close to the maximum volume fraction of \( \phi = 0.64 \) for random packing of spheres. Higher concentrations lead to even higher volume fractions. For the highest used concentration, \( c = 80 \text{ mg/ml} \), we get \( \phi \approx 2 \), which is higher than the close packing fraction. In this case, as mentioned above, the liposome suspension no longer remains entirely confined to the cut-syringe section, and the estimate is not a correct reflection of the actual concentration.

A high concentration liposome suspension can be harvested by carefully removing the cut-syringe assembly from the cuvette, as soon as the turbid front reaches the top of the cut-syringe. As indicated in the main communication, the liposome formation is completed by this time (at about six hours) and a mono-disperse distribution is obtained even without waiting for 36 hours for the entire process to complete.
FIG. 7: **Capillary setup of SPI mechanism.** Liposome synthesis in a capillary by the SPI method, shows completion of liposome formation in less than 45 minutes. Here the distance moved by the turbid front is plotted against time. Also shown below are sample images of the vesicle dispersion forming inside the glass capillary at different times.