Liposome stability and formation: Experimental parameters and theories on the size distribution

M. Winterhalter\textsuperscript{a} and D.D. Lasic\textsuperscript{b}

\textsuperscript{a}CRPP/CNRS, Avenue A. Schweitzer, F-33600 Pessac, (France) and \textsuperscript{b}Liposome Technology, Inc., 1050 Hamilton Court, Menlo Park, CA 94025 (USA)

(Received October 5th, 1992; revision received February 19th, 1993; accepted August 19th, 1993)

The most important characteristics of liposomes, in addition to chemical composition and surface properties, are size distribution and lamellarity. Liposomes can be formed by many different preparation techniques, which according to the literature yield rather well defined vesicle preparations. In contrast to abundant information on the experimental procedures and preparation protocols the theoretical understanding of these processes is lacking. Only geometrical models of structural changes exist for few preparation procedures and size of the liposomes prepared by sonication and detergent depletion method were estimated using simple models. In this paper we first outline different theories on the stability of liposomes and their influence on the size distribution. In the experimental section we shall briefly present the importance of size distribution in experimental work and influence of various experimental parameters on the size distributions obtained.

Key words: vesicles; stability; formation; size distribution; thermodynamics

Introduction

Most applications of liposomes require certain macroscopic features like size distribution, lamellarity or surface properties \cite{1,2}. A typical example for such a limitation comes from the pharmaceutical side using liposomes as artificial drug carriers. Prior to any clinical application their requirements include: (i) a very narrow size distribution and (ii) long-term stability. Vesicles are formed via several metastable states and are in general not thermodynamically stable \cite{3}. For example: gentle hydration of the lipids followed by swelling in excess water \cite{4} yields an ensemble of vesicles with a broad size distribution. Squeezing such a solution through an extruder causes a fairly narrow spectra of vesicle radii. Sonication on the other hand provides a different distribution. It is clear that an equilibrium distribution should yield only one unique and reproducible distribution, independent of the way of formation. In practice, however, the same lipid composition can yield a variety of liposomes with different physical characteristics. By definition one distinguishes large multilamellar vesicles (MLV) and large or small unilamellar ones (LUV and SUV, respectively). Many real samples, however, may be a mixture of all these types, including also giant uni- or oligo-lamellar liposomes (GUV and GOV, respectively).

In this paper we first point out two different theoretical approaches to describe the experimental observation of stability and size distribution. One is based on a thermodynamical approach on a molecular level. The other one uses a macroscopic picture and describes the vesicles in terms of material properties. In the second part techniques for liposome preparation will be discussed. We shall briefly review the parameters affecting the liposome size distribution and theoretical foundations and present the most important factors.
Stability of vesicles — Concept of curvature energy

The first attempt to describe the vesicle size distribution started from their thermodynamical equilibrium properties. According to Tanford [5] the lipid molecule has to be in equilibrium with both the aqueous and the lipid phase. Let $\mu_0$ be the chemical potential of a lipid molecule in the aqueous phase and $\mu_1$ in the lipid phase. The probability in finding an aggregate containing $N$ molecules is $\omega(N) = 1/<N_0> \exp{-N(\mu_0 - \mu_1)/kT}$ which is a simple exponential distribution with $<N_0>$ as the mean aggregation number. Obviously such aggregates are energetically favoured if the difference in the chemical potentials $\mu_0 - \mu_1 < 0$. The corresponding distribution for the vesicle radii $R$ is easily obtained knowing that $N = 2.4\pi R^2/d_0$ with $d_0$ as the area per lipid molecule.

$$\omega(R) = \frac{R}{R_m^2} \exp - \left( \frac{R}{2R_m} \right)^2$$

(1)

$R_m$ corresponds to the maximum in the probability distribution. In Fig. 1a we plotted the size distribution for a sample with its maximum at 50 nm. Obviously if the energy difference $\mu_0 - \mu_1 > 0$ the aggregate formation is unfavoured and no maximum in the size distribution is achieved. The predicted size distribution reflects the relation between the energy of condensation and entropy. This model could explain the shapes of the particles, but again predicted a narrow and well characterized distribution for each lipid composition. The model presented so far does not include any preference for a single lipid molecule to aggregate into a vesicle of a certain size. Or, in other words, one may think on geometrical constraints, e.g. the lipids behave like an ensemble of wedges obviously preferring a specific number of molecules to optimize their interaction [6]. Such a model can explain the assembly of asymmetric amphiphiles into micelles of different shapes, either planar, spherical or cylindrical. Such an ansatz yields a very sharp size distribution. However, liposomes are made up of two monolayers which are supposed to be symmetric and therefore give vanishing asymmetry. Another possible generalisation would be to consider a decrease in condensation energy with increasing aggregate size.

If the aggregates are large enough, it makes sense to consider material properties and use those to describe the equilibrium in an elegant way. Although such approaches are made from totally different points of view, it turns out that both describe the same properties [7]. Namely the curvature [8,9] model becomes a powerful tool to describe many features of large vesicles. In the following we will point out the necessity to introduce higher order elastic terms and later to show their effect on the size distribution.

Everybody understands the shape of soap bubbles. Surface tension causes the film to adapt a minimal surface, which is in general spherical. Considering now the similar case of a spherical stretched vesicle. The material parameter involved is the area stretching modulus $k_E$ and relates the surface tension $\sigma$ to the relative change in area $\sigma = k_E(\Delta A/A_0)$. Evans and Needham [3] have shown that this linear relation between stress and deformation $\Delta A/A_0$ is valid even to the point of failure. The corresponding elastic energy stored per membrane area is

$$f_c = \int_{A_0}^{A_0 + \Delta A} \sigma dA = k_E \left( \frac{\Delta A}{A_0} \right)^2$$

(2)

Let us imagine that we increase the osmolarity of the solution, e.g. by the addition of sucrose. In
other words, due to the low permeability of the liposome, we lower the surface tension by osmotic deflation. Entropy will now cause the interface to undulate and finally the membrane interface will disappear. However, experimental observations [10,11] of undulating vesicles show rather well defined shapes. Within the Hookean limit this behaviour can be attributed to second-order elastic moduli. Such a model has been derived by Helfrich [8] almost 20 years ago. Starting from an analogy with the elasticity of smectic liquid crystals the energy per unit area is

\[ f_c = \frac{1}{2} k_c (c_1 + c_2 - c_0)^2 + \overline{k} c_1 c_2 \] (3)

where \( c_1 \) and \( c_2 \) are the two principal curvatures. This model implies three different elastic parameters, \( k_c \) is the bending modulus, \( \overline{k} \) the Gaussian parameter and \( c_0 \) stands for the spontaneous curvature, showing that due to an intrinsic asymmetry the state of lowest energy may not be the planar one. Standard lipid vesicles consist of two individual monolayers of equal composition. Although the lipid molecule itself can have an intrinsic asymmetry, a bilayer made up of two of these will show no spontaneous curvature. For spherical symmetry the two principle curvatures simplify to \( c_1 = c_2 = 1/R \). Integrating Eqn. (3) over the entire surface of a sphere yields the total curvature elastic energy

\[ F_c = 4\pi(2k_c + \overline{k}) \] (4)

Obviously this expression is independent of radii and the sign depends on the sum of the two elasticity moduli. The bending modulus \( k_c \) for lipids is in the range 2.5–25 \( kT \) (or 0.2–2 \( \cdot 10^{-19} \) J), whereas the Gaussian modulus \( \overline{k} \) has not yet been measured. However theoretical estimation [7] and experimental observation suggest that it should be negative and of about the same value. Following an earlier calculation by Helfrich including \( F_c \) in Eqn. (2) gave raise to an additional term in the difference of the chemical potential. However, as this additional term contains no explicit size dependence, it is to be included in the pre-exponential factor and leaves the size distribution unchanged.

In the following calculations we apply recent theoretical estimations [12] of the moduli to predict the stability of charged liposomes. Although the membrane is made up of a mixture of individual charged and neutral lipids the electrostatic contribution is well described by a uniform charge density \( \sigma \). The Poisson-Boltzmann equation is used to calculate the counter ion distribution. The non-uniform charge distribution will cause an additional pressure in the aqueous phase. Via an analogy to mechanics one can deduce the electrostatic contribution to the bending modulus

\[ k_e = \left( \frac{2k_BT}{e} \right)^2 \epsilon_\omega \lambda_D \frac{(q + 2)(q - 1)}{q(q + 1)} ; \]

\[ q = \sqrt{1 + \left( \frac{\sigma e \lambda_D}{2e_\omega k_BT} \right)^2} \] (5a)

where \( k_B \) is the Boltzmann’s constant, \( T \) the temperature, \( e \) the elementary charge, \( \epsilon_\omega \) is the dielectric permittivity of the aqueous phase. The ion density \( n_0 \) is related to the Debye length \( \lambda_D = \sqrt{2e_\omega n_0 e_\epsilon k_BT} \). Inserting reasonable values found for liposomes proves that for large Debye lengths the electrostatic contribution can be dominant.

Inspection of the above equation shows, that the scaled effective charge \( q \) for a reasonable Debye length becomes greater than 1 even if the charge density \( \sigma \) represents only a few % of charged lipids. In other words, in this case the electrical contribution to the bending modulus \( k_e \) itself reaches its plateau value even at relatively small densities and increasing the amount of charged lipids will show no further increase of the rigidity. More important however is the influence of the charge density on the Gaussian modulus

\[ \overline{k} = -2 \left( \frac{2k_BT}{e} \right)^2 \epsilon_\omega \lambda_D \int_1^{1+q} \frac{\ln z}{z-1} \, dz \] (5b)

In contrast to the electrostatic contribution to the
bending modulus the Gaussian modulus is unbounded and diverges logarithmically with increasing charge density. Adopting Eqn. (4) for the total energy of a vesicle but for only the electrostatic contribution and plotting as in Fig. 2 the total elastic energy versus Debye length we find a clear cross-over. Depending on the Debye length the total energy contribution becomes negative above a certain charge density which will cause spontaneous vesiculation. As the total bending energy of a liposome, is within the curvature model, size independent, the process of vesiculation will create smaller and smaller vesicles until higher order elastic terms can cause stabilisation. Such an experiment can be used to measure/estimate Gaussian modulus.

The curvature model so far presented is based on a homogeneous composition of lipids. The spontaneous curvature \( c_0 \) accounts for an asymmetry of the entire membrane, if the inner monolayer and the outer monolayer are different. This can be achieved e.g. by titration of liposomes [13]. The usual swelling of lipids from a hydrated phase causes both membrane monolayers to be homogeneous and although the monolayer can be asymmetric the bilayer becomes symmetric with vanishing spontaneous curvature. However, this model can be generalized in a way that can account for a possible demixing of a two- (or multi-) component lipid mixture. Consider two different types of lipids, e.g. 1 and 2 with their respective spontaneous curvatures \( c_0^1 \) and \( c_0^2 \). If they do not show a specific interaction between different types of molecules, or in other words, if the average bond distance between the headgroups does not alter, then the planar geometry is always that of lower energy. In the case of a specific interaction, however, the average headgroup distance will depend on the concentration of the lipids. If this change in energy overcomes the entropy of mixing this will cause an asymmetric bilayer. As Safran et al. [14] have suggested this may cause a different spontaneous curvature in each monolayer. Applying again the curvature model for each monolayer separately this may lower the energy of a vesicle and for a certain radius of curvature the bending energy of a vesicle can indeed be lower than the one of planar geometry. For such vesicles size distribution should be independent on the history and dependent on the exact molecular structure of the amphiphile and on medium conditions. In such systems much better prediction can be made and then tested.

An example for this [15] has been observed for a vesicular phase of mixed surfactants containing positively and negatively charged headgroups. This process may also be the reason for the stability of liposomes containing lipids with an attached polymer at the headgroup [16]. If the density of polymer-attached lipids is sufficiently high then the individual polymers may feel each other which gives rise to a larger headgroup area. A slightly larger number of polymers in the outer monolayer, which can be induced during preparation, will stabilise the vesicle.

Recently, investigations of the stability and the shapes of vesicles within the curvature model has again been found of interest by several groups [17–19]. Because of the low permeability of the membrane and as water is incompressible, one assumes constant volume. Furthermore the large value of the area stretching modulus \( k_E \) will cause constant area. Numerical integration of Eqn. (3) under these constraints give the shape of minimal energy. The great variety of possible shapes and their transition is demonstrated in several phase diagrams [17–19]. Starting e.g. from a given asym-
metry represented by a spontaneous curvature $c_0$ and increasing the excess area gives a great variety of shapes of minimal curvature energy but well defined for each point of the phase diagram. Within the curvature model it can be shown that depending on the spontaneous curvature larger excess area causes budding and finally vesiculation. Some of the theoretical predictions have been nicely and experimentally verified [10]. It has been shown that the change in spontaneous curvature can be induced by adding asymmetric amphiphiles, changing the osmolarity in the outer aqueous phase or varying temperature.

The curvature model as presented by Eqn. (3) does not include thermal fluctuations of the interface. Accounting for them causes a decrease in total elastic energy which depends on the specific macroscopic size. Large vesicles appear to be more flexible. The bending modulus $k_c$ as a material parameter has to be replaced by an observable parameter, $k_{eff} = k_c - kT/8\pi(\ln N/2)$. Following again the calculation by Helfrich yields a different size distribution [9].

$$\omega(R) = \frac{9R^3}{2R_m^4} \exp(-\frac{3R^2}{2R_m^2}) \tag{6}$$

In Fig. 1b we again included the corresponding plot for a distribution with its maximum at $R = R_m$. Accounting for interfacial fluctuation yields a size-dependent contribution to the energy of aggregation. This causes an important narrowing of the size distribution.

The underlying approximation of the curvature model is that the relaxed state is almost planar. However, this model may also be used in cases where the radius of spontaneous curvature becomes relevant in comparison to the membrane thickness. Special care in relation to thickness effects and coupling with other deformations must be taken. Other limitations are that if the deformations become large then non-linear terms must be taken into account.

**Vesicle preparation methods**

Liposomes can be produced by many different techniques which often seem to have nothing in common. Only recent work has shown that a strong correlation and many similarities between different methods exist [20]. It was shown in all cases except in a few exceptions of spontaneous vesiculation, that external energy is required to induce curvature [21].

This can be done in two ways; (i) by fragmentation of pre-existing bilayers. Each fragment exposes at the edge hydrophobic part of lipid against hydrophilic environment. This unfavorable edge energy is lowered by reducing the edge length by curving up and induces curvature. (ii) Induction of curvature by changing solubility conditions. This can induce curvature either in the hydrophilic part of the bilayer due to asymmetric interactions in the polar head region of two opposing monolayers or in the non-polar part of the bilayer.

The latter is the driving force in the so-called demulsification procedure which, in contrast to most other methods which are 'oil-in-water' systems, starts from water in oil (double) emulsions. Upon depletion of the organic solvent the strong negative curvature of inverse micelles relaxes until inversion of the phases occurs. Subsequently, upon breaking of the gel and further reducing the ratio organic solvent/water vesicles are formed. Analogue, which could occur spontaneously in the case of very soft bilayers ($k_c kT$) would be entropy driven disintegration of the disordered sponge phases into liposomes upon dilution. Asymmetric interactions, on the other hand, induce curvature due to different expansion capabilities in the two monolayers in a bilayer and this results in budding off of smaller vesicles.

We shall now briefly discuss the mechanism of formation.

**Fragmentation**

A typical example of this process is sonication or homogenization of MLV in which the input of external energy results in tearing lamellae into small bilayered flakes which, upon possible fusion, self-close into SUV and eliminate the unfavorable exposure at the edges. According to Helfrich [22] this process is demonstrated by an interplay between the unfavorable edge energy and bending of the flakes. The unfavorable edge energy is proportional to the circumference $2\pi a$ of the flake and
some constant $\gamma$ called line tension. Assuming that at the beginning the flake is flat any curving up will reduce the circumference but induce curvature. If the radius of the fragments is larger than a critical radius $a > a^* = 4(2k_c + k)\gamma$ then this process is favoured.

**Budding off**

Bilayers due to stresses within the membrane or across it can change vesicle shape. The first process which is normally observed in thermal expansion of GUV and which can easily be observed under a microscope is a consequence of the expansion of the bilayer. Changes of the surface area at fixed volume over 3% upon changing temperature or osmotic pressure result in instant destabilisation of the vesicle shape with immediate reclosure and appearance of daughter vesicles [4,10,23]. Slower changes and in particular asymmetric membrane changes result in budding off, either externally after growth of protrusions or internally after invagination. This process occurs in various different vesicle formation methods, including pH changes and with the addition of micelle-forming molecules. It is also the driving force for the growth of myelin figures from dry lipid films. No systematical theoretical analysis has yet been performed. Some correlations between estimated area changes of polar headgroups at different values of pH and hydration and changes of outer monolayer upon insertion of a known fraction of micelle-forming lipids can be found. Some of these transformations were quantitatively calculated for GUV where normal shapes and their dynamics, including topological changes can be calculated by minimization of curvature energy, as defined by Eqn. (3) as a function of parameters such as fixed volume, fixed area, fixed difference in outer and inner monolayer area or different expansion capabilities of each monolayer.

Not much is known, however, about how these calculations scale to smaller vesicles in which curvature radii are too small to support budding off without depletion of membrane material from the parent membrane to such an extent that non-linear curvature moduli would require consideration. (i.e. how does a 60-nm vesicle bud off 30-nm vesicles?).

**Reverse phase**

A qualitative picture of the coalescence of reverse micelles after the removal of organic solvent which then transform into LUV upon the addition of an aqueous phase was proposed [24]. Similar arguments were used to explain the appearance of multilamellar liposomes by this technique. No theoretical approach has yet been tried. The intermediate structures are undoubtedly gel phases which may resemble bi-continuous emulsion, lamellar phases such as L$_3$, or simply a cluster of reverse micelles covered with a monolayer of lipids or flocule of a hexagonal II phase. The high encapsulation efficiencies are related to the high retention of compounds dissolved in the primary aqueous phase and this probably rules out the existence of bi-continuous gel networks.

Figure 3 shows a cryoelectron micrograph of liposomes obtained by different preparative methods. Although both preparations, being of the same composition and concentration, according to the QELS performed on the commercially available instruments, are practically identical (85 nm ± 5 nm), cryoelectron microscopy shows clear differences. Preparation shown in Fig. 3A,C was extruded through 0.4, 0.2 and 0.05 μm filters, while half of the MLV dispersion was microfluidized (see Fig. 3B,D). After 10 passes at 15 000 psi the sample gave the same QELS reading while electron microscopy clearly shows that extrusion gives more homogeneous size distribution. In addition high energies involved in the microfluidization resulted in the formation of micellar structures which can be separated by gel chromatography. This result is not in agreement with many reports in the literature which claim that high energy homogenizations can yield homogeneous size distributions. Obviously, both preparations behave very differently in various processes and their properties, such as stability during further treatments, loading capacity, encapsulated volume and stability have to be taken into account for careful studies and applications.
Fig. 3. Cryoelectron micrographs of two vesicle preparations. The same dispersion of MLV was either extruded (A) or microfluidized (B) to 85 nm, as measured by commercially available QELS apparatus. Figures C and D are the same sample as in Figs. A and B but taken by a negative staining EM. For details see text. Bars represent 100 nm. Courtesy of P. Frederik (A and B) and H.P. Ting-Beall (C and D).
Figure 3A,C also shows that extruded liposomes are, contrary to some expectations and preliminary reports [25], spherical.

**Application of different size distributions**

An attempt to relate the size distribution to intrinsic material properties of the lipid has been made by Tenchov et al. [26]. They considered the size of a vesicle as a (metastable) result of the aggregation of lipid fragments. Clearly such an approach includes a lower limit to size, corresponding to the curving up of such a single fragment. After many independent encounters the statistical distribution for an ensemble of vesicles may be written

\[ \omega(d) = \left( \frac{\delta}{\eta} \right) \left( \frac{d - d_0}{\eta} \right)^{(\delta - 1)} \exp \left( \frac{d - d_0}{\eta} \right)^\delta \]  

(7)

with \( d_0 \) as the minimal size. The two other parameters involved in Eqn. (7) \( \delta \) and \( \eta \) are used as fitting parameters and arise from the process of formation. However, as no model has been given, those parameters are of little practical use. For illustration Fig. 4 shows several distributions showing a maximum at 50 nm.

In recent years a considerable effort has been made in modelling a relation between the kinetics of formation and the measurable size distribution. However, no realistic theoretical model is so far available [27].

The importance of a knowledge of the size distribution of particular preparations in various experiments can be seen from a few simple examples.

As we pointed out in the theoretical section it is rather difficult to predict from the curvature model the exact internal volume of a sample. However, assuming the two different size distributions of Fig. 1. we plotted the corresponding volume-distribution in Fig. 5. The shift of the maxima of each curve is obvious.

Luckily, several techniques exist which offer a very fast and accurate determination of the internal volume of the sample. Experimentally one simply measures the internal volume \( V_i \) and lipid concentration. The agreement with size distribution obtained by a direct method, such as EM, QELS, or gel filtration shows the quality of the preparation. Unfortunately the \( V_i \) determination is normally rather labor intensive and requires the encapsulation of special non-permeating markers and the separation of the non-encapsulated marker molecules. In addition to the leakage problems there is a possibility of the non-even distribution of the marker molecules due to their lower permeability through membranes during swelling. This,
as well as other experimental factors often results in non-spherical, flaccid vesicles which complicate the analysis further.

There are several techniques which can circumvent all these problems. Here we shall mention electron paramagnetic resonance (EPR) spin labeling which is a very convenient method because it does not require any special sample preparation. The sample is simply labelled with an aqueous solution of spin label which partitions very quickly and evenly in aqueous phases. A paramagnetic relaxing agent is added to the external solution and because it cannot permeate through the membrane this broadens only the external compartment and from the ratio of signals after and before bleaching, the internal volume of the sample can be obtained in approximately 10 min [28,29]. Similar studies can be performed also by using fluorescent probes [30]. In samples of liposomes pre-labelled with various hydrophilic markers gel filtration [31] or other separation methods can be used [31].

Discussion

The preceding arguments have shown that in our limited understanding of liposome formation and stability, thermodynamic and mechanical analysis can yield estimates of size distributions only in cases of thermodynamically stable liposomes. Similar treatments can also be applied to single systems with asymmetric electrostatic and/or steric interactions on each side or symmetric membranes asymmetrically titrated with curvature-inducing amphiphiles, polymers or (poly)ions [33–35]. Such theoretical analyses have not yet been performed.

In all other cases, which account for the vast majority of liposome preparation methods, the above explained thermodynamical or mechanical arguments have to be correlated with kinetic considerations [36]. Despite the fact that kinetic factors, due to the limited understanding of the nature of all these processes and their immense complexity haven’t been modelled yet, the theoretical arguments presented above offer some qualitative understanding of the influence of different parameters in various liposome preparation methods on the size distribution of vesicles formed.

References