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Vesicles as an equilibrium structure of a simple surfactant-water system

L. Cantù (1), M. Corti (2), E. Del Favero (1) and A. Raudino (3)

(1) Department of Chemistry and Biochemistry, Medical School, University of Milan, via Saldini 50, 20133 Milan, Italy
(2) Department of Electronics, University of Pavia, via Abbiateggrosso 209, 27100 Pavia, Italy
(3) Department of Chemistry, University of Catania, viale A. Doria 6, 95125 Catania, Italy

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Abstract. — Unilamellar vesicles are observed to form spontaneously in dilute water solutions of the ganglioside GM3, which is a double-tailed biological amphiphile with a saccharidic headgroup. Static and dynamic, both polarized and depolarized, laser light scattering measurements show that the population of vesicles, of about 500 Å in diameter, is in equilibrium with a very small number of much larger non-spherical objects, which can be schematized as discs with an average radius of 5 000 Å. Hydrophobic chains in the vesicle bilayer are likely to show a significative degree of interdigitation. Discs are found to disappear completely when a second amphiphile, GM1, with the same hydrophobic part and a much larger saccharidic headgroup is added. Spontaneous vesicle formation is associated to the small rigidity of the ganglioside bilayer which may derive from the large mismatch in the lateral dimension of the polar heads and hydrocarbon chains.

1. Introduction.

Self-association of amphiphilic molecules into different structures, like micelles, bilayers, vesicles, etc., has been a growing field of interest in statistical physics [1, 2]. Thermodynamic equilibrium is an important condition for quantitative theoretical predictions. Unlike other structures, vesicles are generally believed to be in a metastable state of matter, since preparation methods normally involve supply of external energy, like sonication and pressure filtration, or chemical treatments like detergent depletion or reversed-phase evaporation. Spontaneous formation of small unilamellar vesicles has been nevertheless reported in rather special cases, either by mixing two ionic surfactants with oppositely charged head groups [3, 4], or for unusual pH conditions [5, 6], or by dilution of sponge phases in multicomponent systems [7, 8]. Recently the spontaneous formation of vesicles made of a single amphiphile, the ganglioside GM3, in thermodynamic equilibrium in water at normal pH has also been
reported [9]. This last case is quite different from those reported in [3] and [4] where the interaction between the two different amphiphiles gives rise to a spontaneous curvature of the bilayer and hence the vesicle formation is due to a clear enthalpic effect. In fact, for the single component system the spontaneous curvature of the bilayer is strictly zero since the two monolayers, made of the same amphiphile, have spontaneous curvatures of the same magnitude but opposite in sign. Therefore, entropic effects in conjunction with a quite low bending elasticity of the bilayer play an important role in the GM3 spontaneous vesicle formation.

In this paper a more detailed account of the spontaneous formation of ganglioside vesicles is presented. Careful static and dynamic, both polarized and depolarized, laser light scattering experiments are used to characterize the system and to verify the important finding that in solution vesicles are in equilibrium with a small amount of large aggregates, mostly of lamellar type. Temperature affects both the vesicle distribution and the amount of large aggregates. Thermodynamic equilibrium is quite evident, since large aggregates, after removal by microporous filtration, reform immediately at the expense of the vesicle population present in solution. In addition, if a second amphiphile, of the same type of the ganglioside GM3 but with a larger headgroup, is added, the large aggregates disappear completely and only small unilamellar vesicles of the mixed surfactant type are present in solution. This last experimental observation drives to the non trivial conclusion that the addition of a second amphiphile, which is expected to cover the hydrophobic edges and therefore stabilize finite lamellar objects, has the opposite effect. This discards the possibility that the presence of the large lamellar aggregates in the single GM3 surfactant solutions is due to the stabilization effect of eventual trace impurities which are difficult to rule out from biological amphiphiles. GM3 vesicles are found to have a small bilayer thickness, which can be ascribed to hydrophobic chain interdigitation, a feature which is not normally encountered in phospholipid vesicles.

The behaviour of the pure GM3 ganglioside in solution is qualitatively discussed in terms of the low bending elasticity of the ganglioside bilayer, which may also come from the appreciable mismatch in the lateral dimension of the polar head and the hydrocarbon chains of the ganglioside molecule, with a saccharidic head group much larger than, for instance, the choline of phospholipids. Such a mismatch in packing areas may also justify some anharmonic contributions to the energy of the bilayers. In the case of low bending elasticity, and hence large thermal fluctuations, a small anharmonic term may favour finite size lamellar structures, as shown in appendix. This can help in understanding the existence of a small lamellar population in equilibrium with vesicles in the single amphiphile solution of the ganglioside GM3.

2. Ganglioside molecules in solution.

Gangliosides are sialic acid containing glycosphingolipids that are normally present in the outer leaflet of plasma membranes of vertebrate cells [10]. They have a marked amphiphilic character with an oligosaccharide chain headgroup and a double tailed hydrophobic part constituted mainly by C_{20} sphingosine and C_{18} fatty acid. In nature a large variety of gangliosides is found, which differ in the length and conformation of both the oligosaccharide and hydrocarbon chains.

As an example, figure 1 shows the chemical structure of the ganglioside GM1. A sialic acid A is attached to the sugar II in the oligosaccharide chain I, II, III and IV. Sugar IV is missing in GM2 and sugars IV and III are missing in GM3. Recent NMR studies [11] show that a net of interactions connects rather strongly sugar III and the sialic acid A, thus reducing the overall rotational freedom of the headgroup.

Differently from phospholipids, gangliosides may form micelles since the large sugar headgroup can supply enough curvature to form globular structures rather than vesicles or
Fig. 1. — The ganglioside GM1.

3. Static and dynamic laser light scattering [14].

The laser light scattering technique is not intrusive and, therefore, quite suitable to study equilibrium properties, but it is often believed difficult to be applied to complex distributions of aggregates. The technique is nevertheless very powerful if static and dynamic measurements, both on the polarized and depolarized components, are combined together for a model fitting of the aggregate distribution [15]. Some general expressions for incident polarized light normal to the scattering plane are reported below, in the case of non interacting particles.

3.1 Polarized Scattering. — The excess average scattered intensity \( I_s - I_w \) relative to the intensity scattered by the solvent \( I_w \) can be given in terms of the physical properties of the particles:

\[
\langle I(k) \rangle = (I_s - I_w)I_w = A (dn/dc)^2 \int c(r) M(r) P(k, r) \, dr . \tag{1}
\]

The instrumental constant \( A \) is 5.83 g/cm\(^3\) for water as a solvent and \( dn/dc \) is the refractive index increment of the solution. Polydispersity is accounted for by the term \( c(r) \), which is the weight-concentration of particles of characteristic dimension \( r \) such that \( \int c(r) \, dr = c_t \), the total concentration in g/cm\(^3\) present in solution. \( M(r) \) is the molecular weight, \( P(k, r) \) the particle form factor [16] and \( k = (4 \pi n/\lambda) \sin \theta/2 \) is the modulus of the scattering vector, with \( \theta \) the scattering angle, \( \lambda \) the laser wavelength and \( n \) the refractive index of the solution.
For a polydisperse solution of particles in Brownian motion, the modulus of the field correlation function \(|g_1(\tau)|\), describing the temporal behaviour of the intensity fluctuations of the scattered light, is expressed as a weighted sum of exponentials [14, 16]

\[
\langle I \rangle |g_1(\tau)| = \int G(\Gamma) \exp(-\Gamma \tau) \, d\Gamma
\]  

(2)

where \(\Gamma = Dk^2\), with \(D\) the particle translational diffusion coefficient (interactions are neglected). \(G(\Gamma)\), the intensity of the scattered light at a given \(k\)-value due to particles having diffusion coefficient \(D\), is given by:

\[
G(\Gamma(k, r)) = A (dn/dc)^2 c(r) M(r) P(k, r)
\]  

(3)

where the connection between \(\Gamma\) and the geometrical parameters, here summarized with the characteristic dimension \(r\), is given by the particle hydrodynamics. It is important to stress that both statics and dynamics depend on polydispersity via the concentration distribution \(c(r)\) weighted by the product \(M(r) P(k, r)\). Furthermore, particle shape effects come into dynamics not only by the term \(P(k, r)\), as in statics, but also from the particle hydrodynamics. For instance, the Stokes Einstein relation for spheres

\[
D = k_B T/(6 \pi \eta R_H),
\]  

(4)

with \(k_B\) the Boltzmann constant, \(T\) the absolute temperature and \(\eta\) the solvent viscosity, is extended to ellipsoids with the hydrodynamic radius \(R_H\) given in terms of the Perrin factor [17] \(f\) and the radius of the sphere of equivalent volume \(r_e\) as \(R_H = fr_e\). The Perrin factor is shape dependent. It is obviously 1 for a sphere and increases with the axial ratio with two different functional dependences for oblate and prolate ellipsoids. The translational diffusion coefficient \(D\) of an oblate ellipsoid of rotation was calculated by Perrin [18] in terms of its minor, \(b\), and major, \(a\), dimension and axial ratio \(\rho = b/a\) as:

\[
D = (k_B T/(6 \pi \eta b)) \rho F(\rho)
\]  

(5)

with

\[
F(\rho) = (\rho^2 - 1)^{-1/2} \tan^{-1} [(\rho^2 - 1)^{1/2}].
\]  

(6)

From a measurement of the intensity correlation function, the function \(|g_1(\tau)|\) is obtained on a set of delays. In principle, it is then possible to solve the integral equation (2) for the weighting function \(G(\Gamma)\) and hence the desired distribution \(c(r)\) [14]. In practice statistical and systematic errors in the collected data make the inversion process rather difficult. If shape polydispersity is also present, direct inversion becomes hopeless. In these cases it is useful to characterize polydispersity by the cumulant time expansion of the logarithm of the normalized field correlation function [14]. The linear term

\[
\langle \Gamma \rangle = \int \Gamma G(\Gamma) \, d\Gamma
\]  

(7)

and the quadratic term

\[
p = \int (\Gamma - \langle \Gamma \rangle)^2 G(\Gamma) \, d\Gamma
\]  

(8)

can be obtained experimentally with good precision, so that they can be used profitably in a fit of a model distribution.
3.2 DEPOLARIZED LIGHT SCATTERING. — If aggregates are formed by optically anisotropic molecules, like the ganglioside GM3 for instance, the scattered light can have a depolarized component. Depolarized light scattering theory predicts that for thin spherical vesicles, even if made up of anisotropic molecules, no depolarization can occur, due to internal cancellation effects [19]. On the other hand if the shape of the aggregate is axisymmetric, the optical anisotropy of the molecule gives rise to a depolarization effect. This is the case with an amphiphilic cylindrically-symmetric molecule embedded into a cylindrically symmetric aggregate like a disc. Depolarization measurements are therefore quite useful to evidence fragments of bilayers in presence of a large population of spherical vesicles, because vesicles do not contribute.

For thin anisotropic discs, theoretical calculations [20] predict the angular dependence of scattered light intensity in the VH configuration, that is the horizontally-polarized scattered intensity $I_{VH}$, due to incident light polarized vertically (perpendicular to the scattering plane):

$$I_{VH} = A (9 \delta^2/k^2) [I_2 - I_3 + \sin^2 \theta/2 (1.25 I_3 - I_2)]$$

(9)

with $\delta$ the optical anisotropy, $A$ a normalization constant [18], $h = (4 \pi R/\lambda) \sin \theta/2$. $R$ is the disc radius and

$$I_n = \int J_1^2 (h \sin \gamma) (\sin \gamma)^{2n-3} \, d\gamma .$$

(10)

Moreover, also dynamic light scattering measurements can be made in the VH configuration to obtain information on the rotational diffusion coefficient $\Theta$ of the nonspherical particles, which, as already stated, are the only ones which contribute to the depolarized scattered intensity. For an oblate ellipsoid of rotation, the rotational diffusion coefficient is given by [16]:

$$\Theta = (3 k_B T/16 \pi \eta b^3) \rho^3/(2 - \rho^2) F(\rho) - 1 \sqrt{(1 - \rho^4)}$$

(11)

where $F(\rho)$ is defined by equation (6).

The modulus of the field correlation function in VH configuration is then expressed in terms of the translational and rotational diffusion coefficients as [16]:

$$|g_1(\tau)|_{VH} = \exp [- (Dk^2 + 6 \Theta) \tau].$$

(12)

4. Results and analysis.

After the preliminary observation by electron microscopy that GM3 forms vesicles in water solution [21], extensive laser light scattering measurements, both polarized and depolarized, have been performed.

The ganglioside GM3, prepared as sodium salt, was dissolved in 30 mM NaCl water solution at a concentration of 0.1 mM, which is very dilute (0.119 mg/cm$^3$). NaCl was added to shield Coulomb interactions among vesicles [22]. Vesicles form spontaneously also in pure water or at different salt concentrations. As a test of the fact that GM3 solutions reach thermodynamic equilibrium spontaneously, it has been checked that sonicated and unsonicated solutions give exactly the same results. All measurements reported in this paper have been performed with unsonicated solutions. Solutions were filtered on 0.4 µm polycarbonate filters prior to measurements. Aggregates in solutions were studied as a function of temperature, between 7 °C and 50 °C. The temperature dependence of the average scattered intensity $\langle I \rangle$ and the apparent radius $R_{app}$, measured at the scattering angle of 90°, are reported in figure 2 and figure 3 respectively. The apparent radius is calculated with equation (4) from the
average diffusion coefficient $\langle D \rangle$ obtained directly from the measured first cumulant $\langle I \rangle$ (Eq. (7)). Of course, $R_{\text{app}}$ acquires a real meaning of an average radius only if all aggregates in solutions have spherical shape, which is not the case for the GM3 solution, as it will be shown later. At variance with the case of phospholipids vesicles, measured data do not show any hysteresis effects in heating and cooling runs at different scan rates from 200 to
1 000 s/°C. The average scattered intensity as a function of angle is reported in figure 4 for $T = 7 \, ^\circ\text{C}$ and in figure 5 for $T = 50 \, ^\circ\text{C}$. The variance $v = p \langle \Gamma \rangle^2$, obtained from the measured second cumulant, equation (8), at $\theta = 90^\circ$ as a function of temperature is reported in figure 6.

A first glance at the angular intensity distributions of figures 4 and 5 indicates that a large polydispersity is affecting the system. A steep curve at low $k$ values is typical for large aggregates, while a flatter one is present at high $k$ values, more likely to be due to much smaller particles. This first impression is supported by the fact that $\langle \Gamma \rangle$, the first cumulant, does not scale as $k^2$ and that $v$ is high and keeps growing while reducing the observation angle. This can be the observed behaviour if very different $P(k, r)$'s weight the particle distribution at different

![Fig. 4. — Average scattered intensity (full squares) measured as a function of angle (in units of momentum transfer) for the GM3 solution at a temperature of 7 °C. Open symbols represent the values obtained with the fit (the full line is drawn to guide the eye).](image1)

![Fig. 5. — The same as figure 4 at a temperature of 50 °C.](image2)
Cumulant analysis was observed to give quite reproducible results on repeated measurements, while direct numerical inversion procedures of the correlation function were not. Therefore, a model distribution of aggregates which gave the best agreement on absolute scale with all the measured data was searched by judicious trials.

Vesicles are known to be present in solution [21] by preliminary electron microscope observations. The vesicle molecular weight $M_v$ can be related to its radius $r$, as $M_v = m [2 \pi \ell/(3 V)] (6 r^2 + \ell^2/2)$, with $m = 1$ 195 Dalton the GM3 monomer molecular weight, $V = 965 \, \text{Å}^3$ the monomer hydrophobic volume [23], $\ell$ the hydrophobic layer thickness and $r$ the vesicle radius taken at the middle of the hydrophobic layer. The form factor $P_v(k, r)$ of a vesicle is [22]:

$$P_v(k, r) = \{3[\sin k(r + \ell/2 + s) - \sin k(r - \ell/2 - s) - k(r + \ell/2 + s) \cos k(r + \ell/2 + s) + k(r - \ell/2 - s) \cos k(r - \ell/2 - s)]/[\{(k^2(r + \ell/2 + s)(3 r^2 + (\ell/2 + s)^2))\}]^2$$

with $s = 8 \, \text{Å}$ the thickness of the hydrophilic layer [21]. Since a large range of dimensions has to be covered, a first trial is a Shultz or a lognormal vesicle distribution $W_v(r)$. The result is that it is impossible to reproduce the experimental $\langle I(k, r) \rangle$ with such monomodal distributions, regardless of the fitting parameters involved. This means that an additional distribution $W_a(r)$ of large aggregates is present in solution. There is no way to get rid of these large aggregates, since they are in dynamic equilibrium with vesicles. In fact, when they are filtered out, they reform rather quickly. Not being constrained any more to assign the observed large polydispersity to the vesicle distribution only, it is quite reasonable to assume that the vesicle distribution is the one predicted theoretically by Helfrich [25] for vesicle in thermodynamic equilibrium [26]:

$$W_v(r) = N (2/\langle N \rangle)^2 \exp(-N/\langle N \rangle)$$

(13)

where $N = M_v/m$ is the number of monomers in a vesicle. The only free parameter is the average vesicle aggregation number $\langle N \rangle$. The total aggregate distribution $W(r)$ is then:

$$W(r) = W_v(r) + W_a(r).$$

(14)
The shape and relative concentration of the aggregates belonging to \( W_a(r) \) is searched in such a way to reproduce the whole set of experimental data, equations (1), (7) and (8). Of course, any change regarding \( W_a(r) \) drags back a change also in \( W_v(r) \). For example the explicit form of the first cumulant is:

\[
\langle I'(k) \rangle = (k_B T/6 \pi \eta) \left\{ \int dr [c_v W_v(r) M_v(r) P_v(r, k) k^2/(r + \ell/2 + s)] + \right. \\
\left. + \int dr [c_a W_a(r) M_a(r) P_a(r, k) k^2/R_H] \right\}
\]  

(15)

where \( c_v + c_a = c_t \), the total concentration in the solution, \( c_v \) being the concentration of GM3 which goes into vesicles and \( c_a \) the one which goes into the large aggregates. The hydrodynamic radius \( R_H \) depends on the geometry of the aggregate. A constraint to the fit comes from the conservation of the total concentration of GM3 monomers in solution and in particular:

\[
c_t = \int c(r) \, dr = \int [c_v W_v(r) + c_a W_a(r)] \, dr.
\]  

(16)

The general feature of the fitting procedure is that the set of parameters obtained from the statics does not leave any more freedom to the dynamics, as can be seen by comparing equations (1) and (3). Therefore the dynamics provides a stringent test of the fit. If a distribution of spheres is assumed for \( W_a(r) \), a good fit is never reached. In fact, there is no way to obtain a satisfactory fit unless some large flat objects, schematized as very thin oblate ellipsoids, are considered. The final fit is then obtained with an Helfrich distribution of vesicles \( W_v(r) \) and a \( W_a(r) \) made up of the sum of two \( \delta \)-functions, one of flat bilayers \( W_b(r) \) and one of multilayer liposomes \( W_l(r) \), with \( c_a = c_b + c_t \).

Table I. — Fraction of GM3, in % by weight, for the different types of aggregates at the two extreme temperatures, as obtained from the global fit of the light scattering data.

<table>
<thead>
<tr>
<th>( T , (\degree\text{C}) )</th>
<th>Vesicles</th>
<th>Discs</th>
<th>Spheres</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>96</td>
<td>3.9</td>
<td>0.04</td>
</tr>
<tr>
<td>50</td>
<td>98</td>
<td>1.9</td>
<td>0.06</td>
</tr>
</tbody>
</table>

The parameters of the fit, concentrations and dimensions, are reported in table I for the two extreme temperatures. Good consistency in the fit of the absolute scattered intensity data is obtained with a value of 19 Å for the hydrophobic layer thickness \( \ell \). The full lines in figures 4 and 5 represent the result of the fit for the angular distribution of the scattered intensity at the two extreme temperatures, 7 °C and 50 °C. If a linear variation of the fractional concentrations is assumed for intermediate temperatures, the full lines of figures 2, 3 and 6 are obtained. As a consequence, the fit gives a variation of the average vesicle radius \( \langle r_v \rangle \) as given by figure 7, which shows a transition around 30 °C. Of course, the extremely simple distribution of large aggregates, made of two \( \delta \)-functions, may not be too realistic, but it is surely indicative of the presence of large flat objects in solution. A strong verification of this last conclusion is given by the existence of a depolarized component of the scattered light, which cannot be due to centrosymmetrical aggregates, like vesicles and liposomes. Figure 8 shows the average
Fig. 7. — Temperature dependence of the average vesicle radius resulting from the global fit of the polarized scattering data.

Fig. 8. — Angular dependence (in units of momentum transfer) of the depolarized VH average intensity scattered by the GM3 solution (full squares); the full line represents the expected value for thin discs of 5 000 Å radius.

depolarized scattered intensity in the VH configuration as a function of angle for a 1 mM GM3 solution at a temperature of 25 °C. The full line in the figure is the theoretical prediction, calculated with equation (9), of the VH depolarized scattered intensity of thin discs of 5 000 Å in radius. Fitting with the behaviour for the depolarized intensity predicted for rods [27] is not acceptable. From the ratio between the horizontal and vertical components of the scattered light, the value of the intrinsic anisotropy δ can be found to be equal to 0.02, a very low value which is an index of the optical anisotropy of the single GM3 monomer.

Also correlation measurements of the intensity fluctuations of the depolarized component in the VH configuration confirms the existence of lamellar type aggregates. Figure 9 shows the k-
Fig. 9. — $k$-square dependence of the decay constant (full squares) of the field correlation function of the VH depolarized scattered intensity for the GM3 solution; the full line presents the expected behaviour for a polydisperse solution of thin discs (simulated for simplicity with 70% of discs with radius of 3 000 Å plus 30% of discs with radius of 9 000 Å).

square dependence of the decay constant of the field correlation function $I_{\text{VH}} = Dk^2 + 6 \Theta$ (Eq. (12)). Full squares are the experimental values. They do not lie on a meaningful straight line. Low angle data are better fitted by assuming larger dimensions, while high angle values are likely to be due to smaller discs. This is a strong evidence of polydispersity: in fact, larger objects give a higher contribution to the intensity in the forward direction with respect to smaller particles. The rotational diffusion coefficient measurements are indeed quite sensitive to polydispersity, due to their cubic dependence on the particle radius (Eq. (11)). Of course, this sensitivity is lacking in the measurements of the average intensity of the depolarized scattered light, which could only be used to determine the average disc-dimension of 5 000 Å. The full line in figure 9 is calculated (Eqs. (5) and (11)), with a polydisperse solution of thin discs, approximated as oblate ellipsoids with very large axial ratio. For simplicity, the disc distribution has been approximated with 70% of discs of 3 000 Å radius and 30% of discs of 9 000 Å radius.

It may be argued that the existence of a small population of discs in equilibrium with vesicles is due to the presence in solution of some impurities of amphiphilic nature which could shield from water the unfavourable edges of the discs. In order to stabilize the discs, these molecules must have a smaller packing parameter [2] than GM3, that is a larger ratio of the head-to-tail cross sections. This point has been checked by adding to the GM3 solution a second amphiphile, the ganglioside GM1, which has the same hydrophobic part of GM3 but a larger headgroup. In fact, GM1 by itself forms micelles in solution with a small radius of curvature [11]. The effect is that discs are not at all stabilized, but they gradually disappear as the GM1 concentration is increased, until a pure vesicle solution is obtained for a GM1 to GM3 ratio of 35 to 65. This is shown in figure 10, where the angular distribution of the scattered light intensity of the mixed ganglioside solution is shown together with the pure GM3 solution, for comparison. The large scattered intensity at low angles disappears, when GM1 is added, which is a clear indication of the absence of large aggregates. Besides, the scattered depolarized light intensity is observed to drop down completely, which is a further verification that discs are no more present in solution. The temperature dependence of the mixed vesicles average radius is also completely different (Fig. 11). Absence of impurities in the biologically-
Fig. 10. — Angular distribution (in units of momentum transfer) of the polarized average scattered intensity of the mixed GM1-GM3 solution in the molar ratio 35 to 65 for a total concentration of 1 mM (full triangles). The angular distribution for the pure GM3 solution is shown for comparison (full squares).

Fig. 11. — Temperature dependence of the average hydrodynamic radius of the GM3 : GM1 = 63 : 35 mixed ganglioside vesicles.

prepared ganglioside GM3 is also confirmed by the fact that test experiments performed on a small sample of synthetically prepared GM3 [28] gives exactly the same scattering curves as the ones obtained with the biological sample. GM3-GM1 solutions have been prepared first in chloroform-methanol, so that gangliosides are dissolved and mixed in monomeric form, dried under vacuum and than dissolved in water.

The light scattering result that the hydrophobic layer thickness of GM3 vesicles is small is quite interesting, as it can be due to chain interdigitation. In fact, the best fit value is close to
the length of the hydrophobic part of a single GM3 ganglioside molecule and not twice as large as it would have been expected if GM3 vesicles behaved like normal phospholipids ones. Small-angle X-ray measurements [29] performed on the same GM3 vesicle solution seem to confirm such a small bilayer thickness.

5. Interpretation and discussion.

The experimental data given so far for the ganglioside GM3 solutions present interesting results: i) vesicles form spontaneously in a single amphiphile water solution and are thermodynamically stable, ii) the bilayer thickness is smaller than twice the GM3 molecular length, meaning chain interdigitation, iii) different populations of aggregates are found in solution, that is, a few percent of the amphiphilic molecules does not go into vesicles, but forms larger aggregates mainly of disc-like shape. iv) the addition of a second amphiphile, the ganglioside GM1, with a larger headgroup than GM3 does no stabilize discs, as it could be expected if GM1 provided a better coverage of the disc edges. Instead, the large aggregates progressively disappear and finally, only vesicles are found to be present in the mixed solution.

The rather unusual features of these experimental findings justified the effort of obtaining independent consistency checks, like the static and dynamic depolarized light scattering measurements for the existence of lamellar-type aggregates, and the small angle X-ray measurements for the bilayer thickness.

The fact that GM3 vesicles form spontaneously in solution is a clear indication that the energy cost to form a vesicle is rather small, of the order of the thermal energy. For a symmetric bilayer the bending energy per unit area [30], to the approximation of no higher terms than second order in the mean curvature \( H = c_1 + c_2 \) contributing to it, may be written as \( g = \frac{1}{2} \kappa H^2 + \bar{K} K \), where \( K = c_1 c_2 \) is the Gaussian curvature of the bilayer, \( c_1 \) and \( c_2 \) being the principal curvatures. There are two elastic moduli, the bending rigidity \( \kappa \) and the modulus of Gaussian curvature, \( \bar{K} \). The spontaneous curvature term is absent, for symmetry reasons, since the two monolayers are made of the same amphiphile, the ganglioside GM3. The bending energy of a sphere (\( c_1 = c_2 = 1/r \)) is \( 4 \pi (2 \kappa + \bar{K}) \). It may be regarded as the minimum energy required to form a vesicle from a planar bilayer and it is independent of the vesicle radius, within the above approximation. The bending rigidity modulus \( \kappa \) is positive, while \( \bar{K} \) may be positive or negative. When two monolayers made of the same amphiphilic molecules are stuck together to form the bilayer, each monolayer feels frustrated for any finite value of the spontaneous curvature of the monolayers. As a consequence of the frustration of the monolayers, the modulus of Gaussian curvature \( \bar{K} \) of the bilayer is found in general finite [31] and its sign is just the opposite of that of the molecular asymmetry, defined by the difference of the mean head and chain areas of the amphiphilic molecule, as demonstrated in reference [32]. For the GM3 ganglioside molecule the area occupied by the head group, made of three sugar rings of 4 Å each arranged in a ramified structure, is undoubtly larger than the hydrophobic area determined by the chain spacing, of the order of 4.2 Å [33]. The modulus of Gaussian curvature is therefore expected to be negative for the ganglioside bilayer. There are also good reasons to believe that the bending rigidity itself is small as compared to the one reported normally for phospholipids [34]. First of all the large head group dimension may cause disorder in the hydrophobic chains, then the hydrophobic thickness is small. Both effects are known to reduce the bending rigidity of the bilayer [35]. Finally, bending rigidity can also be reduced by protrusion effects [36]. In fact the ganglioside GM3 does not have a sharp transition from the hydrophilic to the hydrophobic part along the molecule, due to the presence of the NH, CO and OH groups in the sphingosine (Fig. 1) which may allow more freedom to its motion normal to the bilayer surface. A low value of the bending rigidity modulus, of the order
of $k_B T$, has also been found experimentally for large vesicles of glucosidic surfactants [37]. The bending energy of the GM3 vesicles can therefore be quite small, due to the combined effect of a small rigidity modulus and a negative $\kappa$. It is clear that the vesicle phase can be thermodynamically stable in this case, since translational entropy can balance the small positive energy required for the vesicle formation. Renormalization of the bending rigidity due to thermal onduations [38] gives a slight dependence of the vesicle energy from its size. The vesicle size distribution (Eq. (13)) used in the fit of the GM3 experimental data, is calculated with an effective rigidity which takes care of this renormalization effect. The average vesicle radius determined experimentally reduces with temperature going from 265 Å to 245 Å from 7° to 50 °C. This could be due to an increased fluidity of the hydrophobic chains of GM3 or, more generally, to the reduction of the effective bending rigidity of the bilayer with temperature.

The value of the average diameter of the spontaneously-forming GM3 vesicles should be of the order of magnitude of the persistence length [39] of the GM3 bilayer, or, similarly, of the length scale $L$ at which the effective rigidity $\kappa'$ becomes very small:

$$\kappa' = \kappa - [\alpha k_B T/(4\pi)] \ln (L/a) \ll k_B T,$$

(17)

where the factor $\alpha$ is predicted to be either 1 [38] or 3 [40] and $a$ is the bilayer thickness. In fact, an ondulated piece of membrane will be « floppy » and start to make contacts with itself, and eventually close up in a vesicle, at effective rigidities near $k_B T$, but still positive. By taking $L$ equal to 530 Å, the GM3 vesicle average diameter, and $a = 35$ Å its bilayer thickness, the effective rigidity $\kappa'$ becomes zero for $\kappa = 0.22 k_B T$, with $\alpha = 1$, or $\kappa = 0.65 k_B T$ when $\alpha = 3$. These values of $\kappa$ are small, but not unreasonable.

The average vesicle radius has been predicted to be a weak function of the surfactant volume fraction, for spontaneous vesicle formation [7], although this dependence has been measured to be even weaker. The same behaviour has been observed also for GM3 vesicles, for which no appreciable radius variation has been detected over two order of magnitude of concentration values, that is from $10^{-5}$ to $10^{-3}$ in volume fraction of surfactant. The GM3 situation could be indeed similar to the multicomponent system described in reference [7]. If the formalism developed in reference [7], which calculates the average vesicle radius by taking care of renormalization effects both in $\kappa$ and in $\kappa$, is used, a value of $(2 \kappa + \kappa) = 2.7 k_B T$ is found for GM3 vesicles, which, again, is a rather reasonable value.

GM3 vesicles form spontaneously in a situation of zero spontaneous curvature of the bilayer. In fact the two monolayers have exactly the same composition, since the solution contains a single amphiphile. The small amount of NaCl and of free monomers of GM3 (of the order of 10^{-8} M) present in solution cannot alter this picture. Furthermore, it is found that not all amphilic molecules go into vesicles. A few percent of them form larger aggregates (Tab. I) which are in equilibrium with vesicles. This should somehow be connected to the frustration of the bilayer in the vesicles. The thermodynamic equilibrium is out of question, since large aggregates reform very rapidly, after removal by microporous filtration.

It was not possible to quantify the lifetime of this kinetic effect with the light scattering apparatus used in the experiments, but it is surely shorter than 20-30 s, the time required by the scattered intensity to reach its asymptotic value after filtration. It is also out of question that the majority of the large aggregates are flat with some axisymmetric shape. This is clearly demonstrated by the depolarized static and dynamic light scattering experiments. For simplicity, data are fitted with oblate ellipsoids with the major semiaxis of 5 000 Å and an axial ratio $\rho$ of the order of 100. In order to have a general good fit of all data including the scattering intensity at very forward angles, also a quite small number of bulky aggregates has to be included. They represent only about $5 \times 10^{-4}$ of the total GM3 concentration (Tab. I),
which in terms of volume fraction of GM3 in solution is an extremely small quantity, of the order of $10^{-8}$. Aggregates should be bulky, otherwise absolute intensities and dynamic data do not match together. A spherical shape with a radius of $5000 \text{Å}$ has been considered. The dynamic depolarized light scattering data (Fig. 8), indeed indicate that the oblate ellipsoids are polydisperse and not monodisperse as it has been assumed for the fit. Of course the light scattering picture for the large aggregates is necessarily oversimplified, but it is certain that a small population of lamellar aggregates (oblate ellipsoids), with some multilayer stacks (bulky spheres), are present in solution together with the major vesicle population. The thermodynamic understanding of these experimental observations is not at all clear at this moment. Anyway, some energetic considerations can be done. The existence of membrane bunches in solution seems not to be a theoretical problem [41, 42]. The bulky spheres could therefore represents onions of GM3 bilayers which are shown to exist if a fourth order term in the mean curvature is added to the usual bending energy of a closed bilayer of spherical shape [42]. The number $n$ of skins in the onion is then predicted to be inversely proportional to the usual term $(2\kappa + \kappa')$, containing the elastic moduli of the GM3 layers. Since it has been shown above that this term has to be small in GM3 solutions, onion like stacking could be feasible.

It is by far more difficult to accept the idea that lamellar aggregates are thermodynamically stable in solution. The problem comes from the unfavourable edge energy due to water exposure of the hydrophobic chains. In principle, if the elastic energy required to close a vesicle equals the edge energy of the bilayer disc of equivalent surface, the thermodynamic equilibrium between discs and vesicles could be possible [43]. For instance, the edge energy of the GM3 vesicle-equivalent disc of $500 \text{Å}$ radius and $19 \text{Å}$ of hydrophobic layer thickness becomes $10k_B T$ for an edge tension of $2.5 \times 10^{-8} \text{erg/cm}$ which is about a factor of 100 smaller than what it is reported for lecithin vesicles [43]. Such a low value makes the single vesicle-single disc coexistence quite unfeasible. If on the other hand one considers the ensemble of discs and vesicles, the overall edge energy required by discs for a fixed amount of amphiphile going into discs (say, the 2-4% of GM3 of the present case), is inversely proportional to the disc radius. Therefore « infinite » lamellae could be in equilibrium with vesicles, at the limit. In other words, the total positive elastic energy required by the GM3 vesicles, due to the lack of spontaneous curvature of the bilayer, may be equal to the total edge energy that is needed by the lamellar aggregates in the water solution. As already mentioned before, this unfavourable energy condition could be balanced by translational entropy.

It remains to be discussed however why discs of finite dimensions, average radius of 5000 Å, are stable in solution. Again entropic effects could play a role: of course, there is an entropic gain if an « infinite » lamella breaks up into smaller discs. It is interesting however to consider the possibility of having a really small edge energy due to the peculiar features of the GM3 bilayer. The ganglioside molecule itself has a large saccharidic headgroup which is normally believed to be quite hydrated and mobile. It is therefore plausible that the sugar groups could rearrange themselves in order to better shield the exposure to water of the hydrophobic tails at the disc edges. This is even more plausible considering the fact that the hydrophobic layer of GM3 bilayers is already found to be liquid like and thinner than for normal phospholipids due to chain interdigitation.

Another possible way to imagine a reduction of the edge energy is to consider the thermally excited oscillations of a piece of membrane in presence of anharmonic contributions to the energy of the bilayer. It is shown in Appendix that in this case the averaging over all the thermally excited oscillations gives the real structure of the amphipilic assembly somewhat different from the one expected in absence of oscillations. This effect is not found with the usual harmonic approximation. The calculation shows that the anharmonic term introduces a coupling between the in phase and out of phase thermal oscillations of the two monolayers in
the bilayer so to induce thinning of the bilayer on contour lines determined by some spatial modes. Membranes would therefore break preferentially at these lines giving a finite average dimension to the discs. The anharmonic term takes into account the energy difference between a concave and a convex deformation of the flat membrane surface. It may become important when large amplitude motions are present, like in the case of the loose ganglioside aggregates, and when the amphiphilic molecule itself presents a large mismatch between the hydrophilic and hydrophobic cross sectional areas, like for GM3. It could be interesting to perform neutron scattering experiments which can be sensitive to such large-amplitude fluctuations in the high-

It has been shown in the experimental section that large aggregates disappear completely from the GM3 solution when the second ganglioside GM1 is added, leaving vesicles only. This can be easily understood in terms of a theoretical model developed for mixed copolymer bilayers [44, 45]. The second ganglioside has the same hydrophobic part as GM3 but a longer saccharidic chain. The model predicts that curvature energy favours an inhomogeneous distributions of the two gangliosides among the layers. Therefore, spontaneous curvature readjustements via demixing can give rise to a finite spontaneous curvature of the bilayer, favouring energetically vesicles towards other structures.

Furthermore, the observation that discs disappear when GM1 is added is a clear indication that the simple criticism of disc stabilization by amphiphil impurities is not appropriate. In fact, amphiphilic molecules with larger headgroups could in principle segregate at the disc edges to shield hydrocarbons more easily. Indeed GM1 is one of such amphiphilic molecules, but its effect on the GM3 solution is just opposite. It must be recalled, however, that finite size lamellae stabilization by impurities sequestred at the edges works only at very low water content [46, 47].

The fact that the bending energy of the bilayer is minimized when the two different gangliosides are segregated appears also in the temperature scan of the mixed ganglioside vesicle solution (Fig. 10). The GM3-GM1 vesicles behave like normal phospholipid vesicles. They increase dimension with temperature. This is an indication of a more rigid structure, which is understandable since the bilayer frustration of the simple GM3 vesicles has been released with the addition of the second ganglioside GM1. Besides, also hysteresis effects were found in the temperature scans of the mixed vesicles, while they were completely absent in the pure GM3 vesicle scans. For the pure GM3 solution, the fast equilibration times of the vesicle distribution as temperature is varied and when aggregates reform after filtration is an interesting indication that monomer exchange is not the effect responsible of the equilibrium among the different aggregates. It is known, in fact, that monomer exchange in ganglioside micellar solutions is an extremely slow process [48] connected to the very low solubility of ganglioside monomers (cmc in the micellar language). Indeed pure GM3 vesicle should form and break rather rapidly thus allowing reequilibration among the different aggregates in solution.

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Appendix.

Edge energy of a finite size lipid bilayer.

Averaging over thermally sustained oscillations, in presence of an anharmonic contribution to the energy of the bilayer, gives an average structure which is somewhat different from the minimum energy one.

It is useful to treat a bilayer as consisting of two oppositely oriented monolayers placed back-to-back and interacting with each other through the lipid hydrocarbon core. The total potential energy \( U_{\text{pot}} \) may be partitioned into two contributions: the bending energy \( U_{\text{bend}} \) of each monolayer and the energy of interaction \( U_{\text{int}} \) between the opposite leaflets. The simple Helfrich ansatz [49] can be extended as follows:

\[
U_{\text{bend}} = \sum_{j = A, B} \int_S \left[ \frac{1}{2} \kappa_j (c_{1j} + c_{2j})^2 + \frac{1}{2} (\delta \kappa_j / \delta \Omega_0) (c_{1j} + c_{2j})^3 + \kappa_j (c_{1j}, c_{2j}) \right] dS \tag{A.1}
\]

\( A \) and \( B \) denoting the upper and lower monolayers, respectively, \( \kappa_j \) and \( \bar{\kappa}_j \) are the elastic moduli for mean and Gaussian curvature, whereas \( R_1 = 1/c_1 \) and \( R_2 = 1/c_2 \) are the two principal radii of curvature. The anharmonic term \( \delta \kappa_j / \delta \Omega_0 \) (\( \Omega_0 \) being the mean curvature of the \( j \)-th monolayer) takes into account the energy difference between a concave and a convex deformation of the flat membrane surface. This term can become important when large amplitude motions are present. Symmetry considerations require that:

\[
\kappa_A = \kappa_B = \kappa ; \quad \kappa_A = \bar{\kappa}_B = \bar{\kappa} ; \quad \delta \kappa_A / \delta \Omega_{0A} = - \delta \kappa_B / \delta \Omega_{0B} = - \delta \kappa / \delta \Omega_0 . \tag{A.2}
\]

Adopting Cartesian coordinates for the \( Z \)-deformation, \( \eta_j = \eta_j(X, Y) \), of the \( j \)-th surface from the equilibrium planar configuration, the interaction between the hydrocarbon chains belonging to the opposite monolayers can be phenomenologically described as:

\[
U_{\text{int}} = 1/2 \int_S G(\eta_A - \eta_B)^2 \ dS \tag{A.3}
\]

where \( G = \kappa_j / h \) is a force constant related to the isothermal thickness compressibility modulus \( \kappa_r \) of the bilayer and its thickness \( h \). The physical meaning of equation (A.3) is that any local deformation (either positive or negative) of the bilayer thickness increases the energy of the system.

The kinetic energy \( U_{\text{kin}} \), with \( \rho_j \) and \( h_j \) the monolayers density and equilibrium thickness respectively (\( \rho_A = \rho_B = \rho \) and \( h_A = h_B = h \)), takes the form:

\[
U_{\text{kin}} = 1/2 \sum_{j = A, B} \rho_j h_j \int_S (\delta h_j / \delta \xi)^2 dS . \tag{A.4}
\]

Introducing the two new variables \( \xi \) and \( \zeta \):

\[
\xi = \eta_A + \eta_B , \quad \zeta = \eta_A - \eta_B .
\]

more suitable to describe in-phase and out-of-phase motions of the monolayers, and minimizing the total energy \( U_{\text{tot}} = U_{\text{bend}} + U_{\text{int}} + U_{\text{kin}} \) with the constraint of volume incompressibility of the bilayer, one obtains the following two coupled differential equations:

\[
\alpha \nabla^4 \xi + \beta \xi + \tau + \delta \xi / \delta \xi^2 = - \gamma F_1(\xi \xi) \tag{A.5}
\]

\[
\alpha \nabla^4 \xi + \beta \xi + \tau + \delta \xi / \delta \xi^2 = - \gamma F_2(\xi^2, \xi^2)
\]
with $\nabla^2$ the Laplacian operator, $\alpha = \kappa / \rho h$, $\beta = 2 \kappa / \rho h^2$, $\tau = 2 \sigma / \rho h$ and $\gamma = [3/(2 \rho h)] \delta \kappa / \delta H_0$ and $\sigma$ is a Lagrange multiplier determined by the bilayer incompressibility condition

$$F_1(\xi, \zeta) = [\delta (\nabla^2 \xi) / \delta X] [\delta (\nabla^2 \zeta) / \delta X] [\delta (\nabla^2 \xi) / \delta Y] + [\delta (\nabla^2 \zeta) / \delta X] [\delta (\nabla^2 \xi) / \delta Y] +$$
$$+ 1/2 (\nabla^2 \xi \nabla^4 \xi + \nabla^2 \zeta \nabla^4 \zeta) \quad (A.6)$$

$$F_2(\xi^2, \zeta^2) = 1/2 \{ [\delta (\nabla^2 \xi) / \delta X]^2 + [\delta (\nabla^2 \zeta) / \delta X]^2 + [\delta (\nabla^2 \xi) / \delta Y]^2 + [\delta (\nabla^2 \zeta) / \delta Y]^2 +$$
$$+ \nabla^2 \xi \nabla^4 \xi + \nabla^2 \zeta \nabla^4 \zeta \} .$$

In a perturbation approach, the zeroth order solutions of equations (A.5) describe the in-phase and out-of-phase deformation of the two opposite monolayers. The in-phase motion is essentially a bending deformation of the whole bilayer leaving unaltered the thickness of the membrane. By contrast out-of-phase motions describe squeezing deformation of the membrane and are responsible of its rupture provided a critical amplitude has been reached. The periodic solutions of the differential equations give bending frequencies $\omega_{b_{n,m}}$ and squeezing frequencies $\omega_{s_{n,m}}$, which are no longer constant, as in the linear case, but change in a fashion related to the magnitude of the non-linear term. The non-linearity of the forcing terms of equations (A.5) couples bending and squeezing modes. Since their frequencies are very different, also their amplitudes may differ considerably. Mode amplitudes can be evaluated by applying the equipartition theorem with $1/2 k_B T$ per mode. The interesting result of the whole calculation is that the first order correction $\xi \langle 1 \rangle$ of the deformation variable $\xi$, which describes the squeezing modes, presents a time-independent solution which represents a permanent deformation of the bilayer:

$$\xi_{nm}(X, Y) =$$
$$= -12 (k_B T / S_0) [h / (\kappa r h_0)] [(n \pi / L)^2 \cos (2 n \pi X / L) \sin^2 (m \pi Y / L) +$$
$$+ (m \pi / L)^2 \cos (2 m \pi Y / L) \sin^2 (n \pi X / L)] \quad (A.7)$$

where $L$ is the length of the membrane edge (assumed to be a square) and $S_0$ is the molecular area. After averaging over all modes, the mean thickness $h_{eff}$ of the bilayer edge $(X = 0$ or $Y = 0)$ is:

$$h_{eff} = h + \langle \xi \langle 1 \rangle \rangle = h \{ 1 - 2 [(\pi^2 k_B T) / (\kappa r h_0^2)] \delta \kappa / \delta H_0 \} \quad (A.8)$$

where terms of higher order than $(S_0 / L^2)^{1/2}$ have been neglected. The thickness of the bilayer increases or reduces depending on the sign of the anharmonic term. The term $\delta \kappa / \delta H_0$ is positive for the GM3 bilayer, since its headgroup dimension is quite large. Therefore contours of reduced thickness of the membrane sheets are possible for the ganglioside GM3. Since the isothermal compressibility modulus $\kappa r$ is roughly proportional to the bending rigidity modulus $\kappa$ [50], it follows that the decreasing of edge thickness is inversely proportional to the square of the bending rigidity modulus. Therefore, for highly deformable membranes like the GM3 bilayers, it can be important even for small nonlinearities. The amplitude of the permanent deformation of the planar membrane, here described, is larger in the short wavelength region and rapidly decreases in the long wavelength limit. Therefore, for appropriate values of the physical parameters of the membrane, these deformations, coupled with the usual time-dependent thermal fluctuations, may cause an « evaporation » of small membrane fragments. It is worth noticing that the thermally excited squeezing modes calculated in the linear approximation are smaller in the short wavelength limit, rapidly reaching a plateau at intermediate or long wavelengths. These opposite trends suggest that there is a preferential size
of the fragments « evaporating » from a very loose bilayer, these effects becoming negligible for tightly bound aggregates.

References

[26] Vesicle distributions different from the one of reference [25] have also been proposed, see for instance Eriksson J. C., Bergstrom M. and Ljunggren S., Prog. Colloid Polym. Sci. 93 (1993) 225. The precision of the data treatment of the present paper is not sufficient to distinguish among them.
[28] This sample has been kindly supplied by FIDIA Spa.