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LASER-LIGHT SCATTERING STUDY OF SIZE AND STABILITY OF GANGLIOSIDE-PHOSPHOLIPID SMALL UNILAMELLAR VESICLES

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The effect of the presence of ganglioside GM1, up to 20% by mol, on the size, stability upon aging, and changes with dilution, of small unilamellar vesicles of egg phosphatidylcholine (EPC) was studied by static and dynamic laser-light scattering technique. Standardised conditions were preliminarily set up for the preparation of small unilamellar vesicles by the sonication-ultracentrifugation method. Under these conditions the presence of ganglioside caused a progressive decrease of lipid concentration in the upper 2.2 ml of high speed supernatant, from the original 9 μmol/ml to 1.3 μmol/ml reached with vesicles containing 20% of GM1. The hydrodynamic radius (R_H), apparent molecular weight (M) and polydispersity index (ν) decreased in the presence of ganglioside from the values of 16 nm, 3.3×10^6 and 0.15, respectively, to 11 nm, 1.9×10^6 and 0.10, in vesicle preparations with 20% of GM1. R_H and ν of EPC vesicles increased by only 3% and 12%, respectively, upon aging at 37°C for 6 h. The variations of the same parameters in vesicles containing up to 15% of GM1 were still lower, indicating that the presence of ganglioside tends to increase the stability upon aging of small unilamellar vesicles. Dilution of vesicle dispersions down to 0.1 μmol/ml caused a modest, but significant increase of R_H, M and ν. The extent of increase was similar for EPC and EPC/GM1 vesicles with a GM1 proportion lower than 20%.

Passage of GM1 containing vesicles on molecular sieve chromatographic columns, caused changes of R_H, M and ν, which were consistent with the dilution operated during chromatography.

Keywords: laser-light scattering; ganglioside GM1; ganglioside-phospholipid vesicles.

Introduction

Gangliosides are normal components of the plasma membranes of mammalian cells [1], and are assumed to play a role in a variety of surface events, such as

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Abbreviations: GM1, $II^3NeuAcGgOse_4Cer$; EPC, egg phosphatidylcholine.

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specific recognition of external ligands and biotransduction of membrane mediated information [2,3].

Lipid vesicles have been used as membrane models for studying the surface behaviour of gangliosides and the mechanism of their interactions with various ligands [4–6]. Particularly, unilamellar vesicles of phospholipids and gangliosides appeared as the most suitable tools for these studies [7–10].

Different methods are available for preparing lipid unilamellar vesicles [11,12] and emphasis was given to the parameters which affect their stability [13,14]. Among them vesicle size and bilayer curvature were described to be crucial and largely responsible for the fusion phenomena which spontaneously occur in unilamellar vesicles smaller than a certain size (about 400 Å of diameter) [15,16].

The effect of the presence of ganglioside on size and stability of small unilamellar vesicles has been only occasionally considered [5]. However important physical properties of unilamellar vesicles, such as molecular packing density, transbilayer distribution, and thermotropic behaviour of the component lipids are strongly dependent on the surface curvature of the bilayer comprising the vesicle wall [17–19]. Moreover these properties likely play an important role in determining or modulating the interaction capabilities of the ganglioside embedded into the vesicle.

With the present investigation we studied the effect of the presence of ganglioside GM1 on the size, stability on aging, and changes with dilution of small unilamellar vesicles of EPC. Vesicles were prepared by sonication of aqueous dispersions of the component lipids, followed by high speed centrifugation and molecular sieve chromatography. Vesicles diameter and size were assessed by static and dynamic laser-light scattering technique, that was already successfully used for similar purposes [5,13,20–22].

Materials and Methods*

Materials

Commercial chemicals were of analytical grade or of the highest purity available. Solvents were distilled before use. Water was freshly redistilled on a glass apparatus. EPC was purchased from B.D.H. (Milan). It was used without further purification, after checking that it showed a single spot when assayed by high performance thin-layer chromatography (silica gel thin-layer plates, HPTLC 60, Merck GmbH, Darmstadt). The solvent system was chloroform/methanol/water (60:35:4, by vol.) 1 h run at 20°C and spots were revealed by exposure to iodine vapours.

Ganglioside GM1 was extracted and purified from bovine brain according to Tettamanti et al. [25]. Its identification, structure analysis and purity were assayed as described by Sonnino et al [26]. The final purity was over 99%. The long chain

*The ganglioside nomenclature in this paper follows that of Svennerholm [23] and the IUPAC-IUB recommendations [24].

base and fatty acid composition of GM1 ganglioside, determined by gas-liquid chromatographic analysis [26] was the following: (a) long chain bases (mol%) – 2-D-amino-octadecan-1,3-D-diol, 3%, 2-D-amino-octadec-4-ene-1,3-D-diol, 57.5%, 2-D-amino-eicosan-1,3-D-diol, 2%, 2-D-amino-eicos-4-ene-1,3-D-diol, 37.5%; (b) fatty acids (mol%) – C16:0, 2.3%, C18:0, 95%, C18:1, 0.8%, C20:0, 1.9%. The preparation of GM1 used was the sodium salt [27].

Ganglioside GM1 was tritium labeled at the C-3 position of unsaturated long chain bases according to Ghidoni et al. [28]. The natural erythro forms, prepared according to Sonnino et al. [29] were employed. The radiochemical purity was over 99% and spec. radioact., 1.5 Ci/mmol. Ultrogel AcA22 resin was purchased from LKB, Stockholm.

Preparation of vesicles

EPC and EPC-ganglioside small unilamellar vesicles were prepared by the method of Barenholz et al. [11] with some modifications which derived from the suggestions of Cornell et al. [16] and were perfected in the course of preliminary experiments. Eighty-four μmoles of lipids (EPC or EPC plus ganglioside) were dissolved in chloroform/methanol (2:1, v/v), then dried with a gentle flow of nitrogen and finally lyophilized. The residue was dissolved with 7 ml of 25 mM Tris–HCl buffer (pH 7.0) vortexed and submitted to sonication under nitrogen with a 150 W MSE sonicator operating at position 9 of the set, and using a 9.5 mm diameter solid titanium probe. The suspension was sonicated for 5 periods, 4 min each, with 1-min intervals between each period. The temperature of the sample was maintained close to 4°C with the aid of an ice-cold water bath.

After sonication the suspension was transferred into a 8 ml centrifuge tube and centrifuged for 2 h at 140 000 $\times g$ in a Spinco L50 ultracentrifuge. The upper 2.2 ml of the supernatant were carefully siphoned off, drawing from the top. Of the drawn suspension 0.2 ml were used for ganglioside and phospholipid determination, the remainder for laser-light scattering measurements. These were carried out within 40 min from the end of centrifugation.

When gel filtration had to follow high speed centrifugation, about 1.4×10^5 dpm of radioactive GM1 were added to the starting EPC-ganglioside mixture, which was processed exactly as described above.

Molecular sieve chromatography

One ml of the high speed upper supernatant was applied to an Ultrogel AcA 22 column (2 × 80 cm) previously equilibrated and eluted with the same buffer employed for the vesicle preparation. Fractions (1 ml) were automatically collected and 100 μl of each fraction were used for the determination of radioactivity by liquid scintillation counting. The fractions bearing radioactivity were submitted to laser-light scattering measurement and to determination of phospholipid and ganglioside content.

Dilution experiments

The effect of dilution on vesicles was studied using the high speed upper supernatant, or the fractions obtained by molecular sieve chromatography. In both cases the mixtures were diluted up to 10-fold with the same buffer used for sonication. The experiments were carried out at 37°C and at room temperature.

Assessment of ganglioside sidedness in vesicles

Ganglioside distribution in the outer and inner layer of the vesicles was determined by the galactose oxidase method previously described [8]. Shortly, a sample of vesicle preparation (carrying about 20 μg GM1 as sialic acid) was incubated with an excess of galactose oxidase, in the absence or presence of Triton X-100, and oxidized GM1 was separated by TLC and densitometrically quantified. Using this method, GM1 ganglioside was showed to be located for 2/3 in the outer and for 1/3 in the inner layer of the vesicle, regardless of the proportion of ganglioside in the vesicle.

Light scattering measurements

The light scattering apparatus was described in great detail by Degiorgio et al. [30]. We simply recall here that it inculdes an argon ion laser, a temperature controlled cell, and a digital correlator. The scattered light is collected at the scattering angles $\theta = 20^\circ$ and 90°. The absolute intensity calibration is performed according to the method described by Degiorgio et al. [30]. The excess intensity of light scattered by a dilute solution of monodisperse particles with a molecular weight M and a gyration radius R_g is:

$$I_s = A\left(\frac{dn}{dc}\right)^2 cM \frac{1}{1 + K_I c} P(\theta) \tag{1}$$

where c is the particle concentration, K_I is a coefficient which describes the effect of interparticle interactions [31] ($K_I = 0$ for an ideal solution), A is a calibration constant, (dn/dc) is the refractive index increment of the suspended particles and $P(\theta)$ is the particle form-factor which, for not too large particles, may be written as $P(\theta) = 1 - K^2 R_g^2/3$, where $K = (4\pi n/\lambda) \sin \theta/2$ is the modulus of scattering vector. For polydisperse solutions the molecular weight in Eqn. (1) is the average molecular weight.

When the particle is composed of two different molecular species, as in the case of mixed vesicles and mixed micelles, Eqn. (1) must be substituted by a more complex formula [32] which takes into account the different optical contrast of the two species. In the case discussed in this article, $dn/dc = 0.148$ for EPC [33], $dn/dc = 0.168$ for GM1, as measured by us. Dynamic light scattering results yield, on the basis of the cumulant analysis, the average diffusion coefficient D and the

polydispersity index ν [34]. The parameter D can be expressed as

$$D = D_0(1 + K_D c)$$

where D_0 is the translational diffusion coefficient of the individual particle and K_D is a parameter which describes the effect of interparticle interactions ($K_D \simeq 0$ for ideal solutions), as already discussed [31,34]. The Einstein-Stokes relation, $D_0 = K_B T/(6\pi\mu R_H)$, allows to derive the hydrodynamic radius R_H, once D_0 is measured and the viscosity μ of the solution is known.

In the case of an unilamellar vesicle with internal radius R_i and external radius R_e, $R_H = R_e$ (apart from a small correction due to the hydration layer) and $R_g \cong R_e(1 - d/R_e)^{1/2}$, where $d = R_e - R_i$. Note also that, by taking $R_g = 15$ nm, $\theta = 90^\circ$ and $\lambda = 514.5$ nm, we obtained $K^2 R_g^2/3 \cong 0.045$, that is the intensity of light scattered at 90° is about 5% smaller than that scattered at zero angle for vesicles having a radius of gyration of 15 nm.

Analytical methods

Ganglioside content was determined as bound sialic acid acording to Svennerholm [35]. Phosphatidylcholine was determined as bound phosphorous according to Bartlett [36].

Results

Preparation of small unilamellar vesicles

The lipid concentration, duration and power of sonication, probe size, duration and speed of ultracentrifugation, which were optimal for obtaining reproducible low-dispersity, small size unilamellar vesicles, were determined in preliminary experiments, checking the different preparations by laser-light scattering. Among the different procedures, that described in the Materials and Methods was chosen as the most reliable.

Using EPC as phospholipid and starting from 12 μmol/ml of lipid mixture and a 25 mM Tris–HCl buffer, the average recovery in the upper 2.2 ml of high speed supernatant was about 9 μmol/ml, that is about 80% of the original value. As shown in Table I, the recovery progressively decreased when ganglioside containing vesicles were prepared, reaching a minimum value, about 16%, in the case of 20% GM1 ganglioside containing vesicles.

Since the preparation procedure of the vesicles was the same for all lipid compositions (in order to make the use of mixed vesicles more convenient for biochemical investigations), whereas the specific volume of GM1 ($\bar{V} = 0.77$ according to Gatt et al. [37]) is considerably lower than that of EPC ($\bar{V} = 0.98$), the observed decrease in vesicle yield with increasing GM1 proportion may be at least partially

TABLE I

RECOVERY OF MONOLAMELLAR VESICLES COMPOSED OF EPC AND DIFFERENT MOLAR AMOUNTS OF GM1 GANGLIOSIDE

GM1/EPC vesicle composition (% of ganglioside)	Total lipid concentration[a] after centrifugation (mM)	Total recovery[a] (%)
0.00	9.48	79.1
3.75	8.64	72.0
7.67	7.92	66.0
12.11	5.95	49.6
17.01	2.90	24.1
20.00	2.00	16.6
23.35	2.16	18.2

[a] Sum of phosphatidylcholine and GM1 ganglioside.

explained by the dependence of the sedimentation velocity on the lipid composition of the vesicle.

Conditions for reliable laser-light scattering measurements

Dynamic light-scattering measurements, performed within one hour from the end of the preparation procedure at a scattering angle of 90°, and a temperature of 37°C, give for a 10 mM EPC vesicle preparation, assuming an ideal solution without interparticle interactions:

$$R_H = (15 \pm 1)\text{nm} \qquad \nu = (15 \pm 3)\%$$

From the measured R_H, we compute a form factor $P(\theta = 90^\circ) \cong 0.95$. Taking into account this value, the intensity data give

$$M = (3.3 \pm 0.5) \times 10^6 \text{ daltons}$$

The quoted uncertainties represent the reproducibility of the results. In the case of M there is an additional uncertainty in the absolute calibration (10%) [30].

Since the procedure for the preparation of small unilamellar vesicles cannot completely avoid the presence of a small number of large structures (multilamellar vesicles?) in the investigated samples, and the effect of large aggregates is considerable at small scattering angles, we consider the light-scattering data taken at $\theta = 20^\circ$ not sufficiently reliable to extract small vesicle properties. Almost all light-scattering investigations of vesicle solutions have indeed been performed at $\theta = 90^\circ$ [13,16]. We shall therefore discuss the 90° data only.

Vesicle size

By using mixtures of EPC and GM1 of various compositions, the effect of GM1 on the vesicle size at 37°C was studied. As shown in Fig. 1, the radius R_H decreases from the 15 nm value of pure EPC vesicles to the value of 11 nm, when 20% GM1 (as % of total lipid mixture) is present. Consistently, M decreases from 3.3×10^6 to 1.9×10^6. The polydispersity index v decreases from 0.15 to 0.10. Measurements were also performed with vesicle preparations having a GM1 proportion in the mixture over 20%. The results obtained showed a drastic tendency to increase of both M and v. However reproducibility of data was poor and the decision was thus taken to investigate this situation separately and by adequate experimental approaches.

Stability of the vesicles upon aging

The stability of the vesicles present in the high speed upper supernatant was assessed by measuring, in a time interval of 6 h following centrifugation, both the average scattered intensity I_r and the hydrodynamic radius R_H. In Fig. 2 we report the results relative to EPC vesicles and to GM1-EPC mixed vesicles containing 2%, 15% and 20% GM1, all at 37°C. The scattered intensity I_r for EPC vesicles

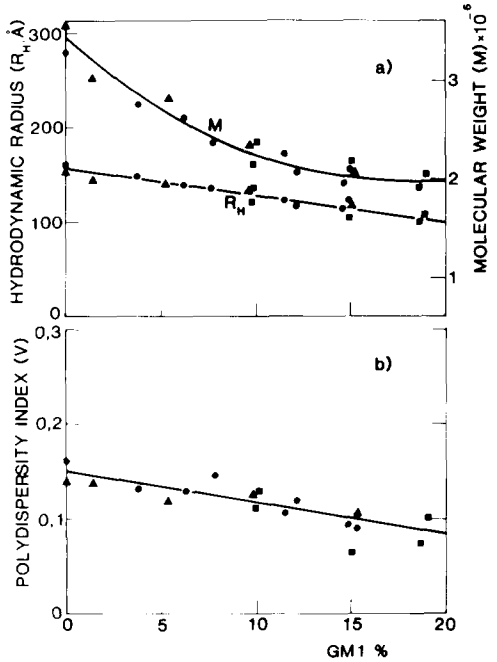

Fig. 1. Molecular weight M, hydrodynamic radius R_H (Fig. 1a) and polydispersity index v (Fig. 1b) as a function of the GM1 molar fraction for EPC/GM1 mixed monolamellar vesicles in 25 mM Tris–HCl, pH 7.0, buffer at 37°C. The various symbols correspond to different series of preparations.

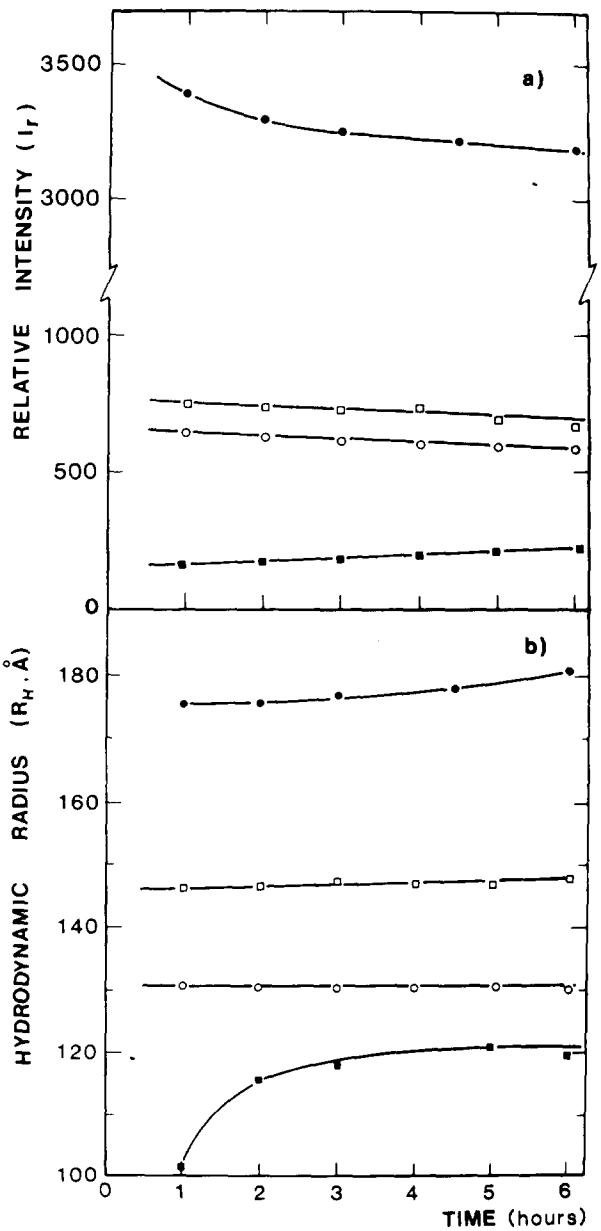

Fig. 2. Scattered light intensity (relative to that scattered by the buffer) I_r (Fig. 2a) and hydrodynamic radius R_H (Fig. 2b), as a function of storage time after preparation, at 37°C for EPC vesicles (full dots) and for GM1/EPC vesicles containing 2% (open squares), 15% (open dots) and 20% (full squares) GM1.

shows a 6% decrease over the investigated time interval, whereas R_H increases of about 3%. The polydispersity index v (not shown in Fig. 2) increases also slightly from 15% to 17%. The data relative to vesicles containing 2% and 15% GM1 follow a trend similar to that of pure EPC vesicles, but the temporal variations are even smaller. Solutions of vesicles containing 20% GM1 show a distinct behaviour because both I_r and R_H increase markedly with time. The stability of the vesicles upon storage is improved, at all ganglioside proportions in the vesicle, by decreasing temperature. For instance, in both EPC and EPC-10% GM1, R_H variations were less than 1% after 5 h at 25°C or 17 h at 15°C. Vesicles containing 20% GM1 were also more stable at temperatures lower than 37°C; R_H-values underwent only a 7% increase after 5 h at 25°C and a 5% increase after 17 h at 15°C. Noteworthy, in all cases temperature decrease from 25 to 15°C, did not affect R_H-values but produced a 5% increase of the I_r-value.

Size and polydispersity of vesicles after molecular sieve chromatography

Figure 3 illustrates the elution profile from Ultrogel AcA 22 column of the EPC-7% GM1 vesicles, present in the high speed upper supernatant, and gives the M and R_H-values along the elution peak, as provided by laser-light scattering measurements. It should be reminded that the lipid concentration, in the eluted

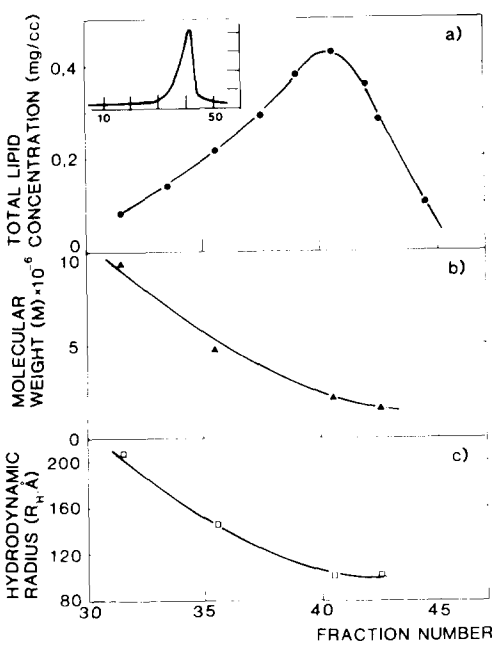

Fig. 3. Molecular sieve chromatography of EPC/7% GM1 vesicles, on AcA 22 column. See Materials and Methods for details. Total lipid concentration (a), molecular weight (b) and hydrodynamic radius (c), are as a function of the elution volume.

fractions was at least one order of magnitude smaller than that of the starting supernatant, indicating the possible occurrence of dilution phenomena (see below).

At the top of the elution peak, where the lipid concentration was relatively higher, M was about 2×10^6 and R_H 100 Å. Both parameters significantly increased in the peak fractions toward lower elution volumes ($M = 9 \times 10^6$ and $R_H = 200$ Å at the beginning of the peak), while tended to be constant moving toward higher elution volumes. In all the fractions constituting the eluted peak the EPC-GM1 ratio remained constant and at the same value displayed by the starting supernatant.

The behaviour on molecular sieve chromatography was similar to that described in Fig. 3 for vesicles containing less than 20% ganglioside GM1. At this and over this proportion rather irreproducible results were obtained in both elution pattern and static and dynamic light-scattering data.

Dilution studies

After having checked the variations of vesicle properties with time and temperature, we investigated dilution effects. An EPC vesicle dispersion high speed upper supernatant, at concentration of 10 mM, was progressively diluted at 37°C, by

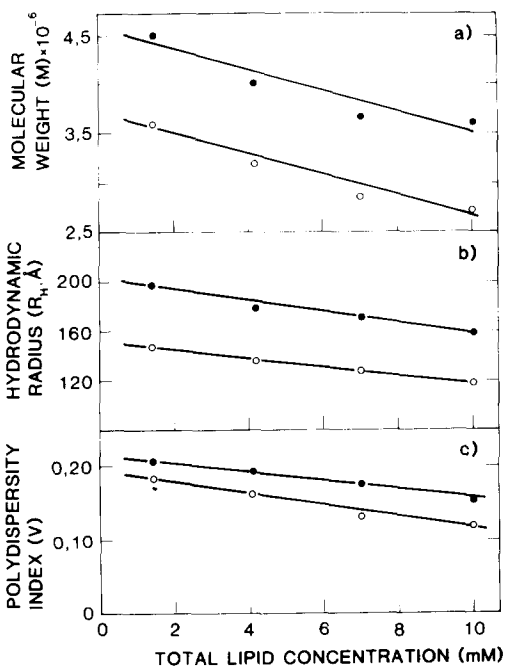

Fig. 4. Molecular weight M (a), hydrodynamic radius R_H (b), and polydispersity index V (c), as a function of concentration for EPC vesicles (full dots) and for EPC/5% GM1 vesicles (open dots) at 37°C. The various concentrations are obtained by successive dilutions of a single vesicle preparation.

adding buffer solution down to a concentration of 1.7 mM. The same procedure was used for a similar EPC-GM1 vesicle dispersion containing 5% GM1. Measurements were performed approximately two hours after preparation. As reported in Fig. 4 both R_H and M increased by increasing dilution, and the same was for polidispersity index. Figure 5a shows data referring to EPC-7% GM1 vesicles obtained after molecular sieve chromatography. Particularly the fractions around the peak edge, containing vesicles of smaller size (R_H = 100 Å), were investigated by progressive dilution down to 0.1 mM. Also in this case dilution caused an increase of R_H, M and polydispersity. In Fig. 5b the data are reported, which refer to EPC-GM1 high speed upper supernatant vesicles containing 20% of ganglioside, progressively diluted to a lipid concentration of 0.2 mM. Both R_H and ν increase considerably upon dilution for this second case. It is evident that in this case the increase of R_H and ν by dilution, underwent a drastic rise below 0.25 mM total lipid.

Discussion

The present communication clearly demonstrates that laser-light scattering is a suitable technique for monitoring size and polydispersity of small unilamellar vesicles of phospholipid and of phospholipid and ganglioside GM1. This confirms and extends previous indications [13,20,22].

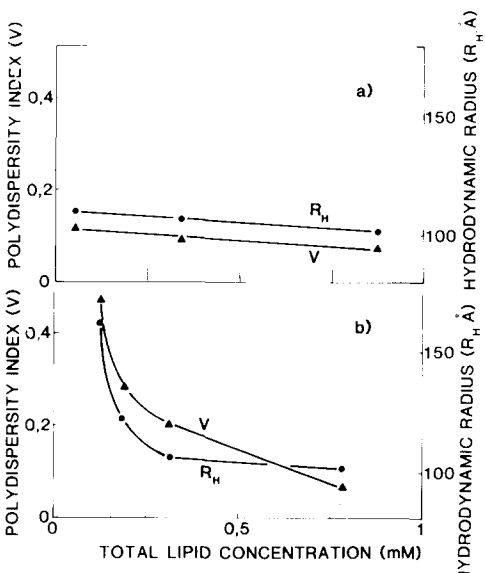

Fig. 5. Hydrodynamic radius R_H and polydispersity index ν, as a function of concentration for a EPC/7% GM1 (a) and a EPC/20% GM1 (b) vesicle dispersion. The various concentrations are obtained by successive dilutions of a single vesicle solution. The sample, which contained 7% ganglioside was submitted to molecular sieve chromatography, before the dilution.

The mutual consistency of the measured hydrodynamic radius and molecular weight for EPC small unilamellar vesicles can be easily verified. Taking into account that the thickness of the lipid bilayer is 37 Å, the hydration layer is 6 Å, the specific volume is 0.985 ml/g and the number of bound water molecules per monomer is 12.4 [38], the expected molecular weight for a vesicle of 155 Å is 3.8×10^6, in good agreement with the measured value of 3.6×10^6. By addition of GM1 the vesicle radius decreases to 105 Å and correspondingly M becomes 1.9×10^6 when the molar fraction of GM1 is 20%. These values are very close to those found by Mason and Huang [38] for the smallest EPC vesicles (R_H = 105 Å, $M = 1.88 \times 10^6$), thus indicating that the addition of GM1 does not produce any appreciable change in the thickness of the lipid bilayer.

A good test of the fact that the lipid aggregates are unilamellar vesicles is represented by the plot in Fig. 6, where all our measurements of R_H and M for EPC and EPC-GM1 vesicles are reported in a log-log plot. The experimental data follow approximately the law $R_H \div M^{1/2}$ as expected for unilamellar vesicles. The scatter of experimental points around the theoretical line is due to uncertainties in the sample concentration and to polydispersity effects.

It is worth commenting that the size of EPC small unilamellar vesicles of 155 Å we obtained is larger than that reported (105 ± 3 Å) by Mason and Huang [38] and by Barenholz et al. [11]. The discrepancy can be likely explained by the shorter time and lower speed of centrifugation of our preparative conditions, besides the differences in the sonication conditions. It was clearly stated in the experimental section that these conditions were chosen in order to obtain not necessarily the smallest vesicles, but a reasonably high and reproducible vesicle yield in the upper supernatant, even with ganglioside-containing vesicles which have a lower specific volume.

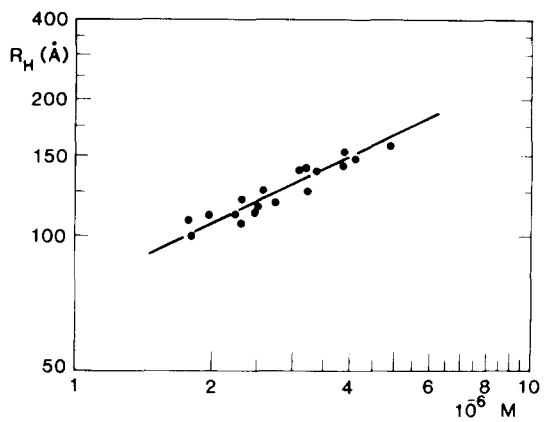

Fig. 6. Hydrodynamic radius R_H vs. molecular weight M for small unilamellar vesicles at various lipid ratios, ranging from 0 to 20% GM1, as determined by dynamic and static light scattering.

A further comment concerns the possible dependence of vesicle size, weight and polydispersity on the presence of ganglioside GM1, since the specific volume of vesicles changes with the GM1 proportion in the vesicles. In order to evaluate, at least approximately, this effect we have performed a calculation on a simple model. We have assumed a trimodal vesicle radius distribution made of 25% of vesicles with radius 110 Å, 50% with radius 150 Å and 25% with radius 190 Å. The average radius of this distribution is 150 Å. In our experimental operating conditions we have calculated the displacement of the sedimentation boundary after a centrifugation time of 2 h for each vesicle size in two cases: pure EPC vesicles and EPC/15% GM1 vesicles. We found that for EPC vesicles the yield is 75% and the average radius 149 Å, whereas for EPC/15% GM1 vesicles the yield goes down to 44% and the average radius becomes 145 Å. The variance of the distribution is very weakly influenced in both cases. We can therefore conclude that the change in specific volume affects considerably the vesicle yield in the supernatant but does not influence appreciably the vesicle size distribution.

As discussed in the Materials and Methods the parameters measured at a given concentration c must be corrected for the effect of interactions. Since all measurements have been performed with diluted dispersions, such corrections are rather small. For instance, by taking a 1 mM solution of EPC vesicles with a radius of 150 Å and assuming that the only interaction effect comes from the excluded volume, the apparent molecular weight is only 1% smaller than the true molecular weight and the apparent hydrodynamic radius is 0.2% smaller than the true one. The dilution data of Fig. 4 follow, for both M and R_H, a trend which could be explained by intervesicle interactions. However, the fact that the polydispersity index is also increasing by increasing the dilution represents an indication of a real (and not apparent) increase of the average M and R_H when the solution is diluted. The dilution data obtained with an EPC dispersion containing 20% GM1 (see Fig. 5b) present very marked variations with dilutions, which are explainable by a real change of the average vesicle parameters. The large increase of the polydispersity shown in Fig. 5b indicates that the effect of dilution is probably that of increasing the rate of irreversible disruption of small vesicles. The disrupted small vesicles are likely to form, by fusion, large structures. The instability of these structures, at total lipid concentration below 0.25 mM is a likely explanation for the abnormal behaviour of vesicles containing 20% GM1 on molecular sieve chromatography. This procedure easily causes a 10–20-fold dilution of the sample.

The temporal stability data of Fig. 2 confirm that dispersions of small EPC vesicles change very little their properties over time intervals of many hours, provided that they are prepared in a very careful and standardised way. The observed small vesicles tend to form irreversibly large structures. Since the scattered intensity is decreasing with time, we conclude that the concentration of lipids in the dispersion, is decreasing with time, that is, the same structures may be so large to sediment in the scattering volume. The temporal stability of EPC vesicles containing GM1 is even better than that of pure EPC vesicles provided that the GM1

fractional concentration is below 20%, as shown in Fig. 2. All the phenomena appearing when the GM1 concentration is above 20% (reduced reproducibility of the results in different preparations, instability upon dilution and aging) can be better understood only after a careful study of the ternary phase dyagram H_2O-EPC-GM1. Indeed some of our observations appear very similar to those reported by Rydhag et al. [39], concerning the system H_2O-dimyristoylphosphatidylcholine (DMPC)-cetyltrimethylammonium bromide (CTAB). These authors reported that above a certain CTAB/DMPC ratio it becomes impossible to prepare stable liposomes because the lamellar phase is in equilibrium with micelles. It is likely that the same situation is present also in our system.

Using the high speed upper supernatant and ganglioside proportions in the vesicles lower than 20%, the changes in size and polidispersity of the vesicles preparations upon aging up to 6 hours and dilution to 0.1 mM total lipid, are relatively modest and anyway known. This will greatly facilitate and make meaningful studies on ganglioside behaviour and interactions at the membrane level, which require operational time and procedural steps involving dilution.

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