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# Aqueous dispersions of DMPG in low salt contain leaky vesicles

Rafael P. Barroso[a], Katia Regina Perez[b,c], Iolanda M. Cuccovia[c], M. Teresa Lamy[a,*]

[a] Instituto de Física, Universidade de São Paulo, São Paulo, Brazil
[b] Departamento de Biofísica, Universidade Federal de São Paulo, São Paulo, Brazil
[c] Instituto de Química, Universidade de São Paulo, São Paulo, Brazil

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ABSTRACT

Aqueous dispersions of dimyristoyl phosphatidylglycerol (DMPG), at low ionic strength, display uncommon thermal behavior. Models for such behavior need to assign a form to the lipid aggregate. Although most studies accept the presence of lipid vesicles in the lipid gel and fluid phases, this is still controversial. With electron spin resonance (ESR) spectra of spin labels incorporated into DMPG aggregates, quantification of [$^{14}$C]sucrose entrapped by the aggregates, and viscosity measurements, we demonstrate the existence of leaky vesicles in dispersions of DMPG at low ionic strength, in both gel and fluid phases of the lipid. As a control system, the ubiquitous lipid dimyristoyl phosphatidylcholine (DMPC) was used. For DMPG in the gel phase, spin labeling only indicated the presence of lipid bilayers, strongly suggesting that DMPG molecules are organized as vesicles and not micelles or bilayer fragments (bicelles), as the latter has a non-bilayer structure at the edges. Quantification of [$^{14}$C]sucrose entrapping by DMPG aggregates revealed the presence of highly leaky vesicles. Due to the short hydrocarbon chains ($^{14}$C atoms), DMPC vesicles were also found to be partially permeable to sucrose, but not as much as DMPG vesicles. Viscosity measurements, with the calculation of the intrinsic viscosity of the lipid aggregate, showed that DMPG vesicles are rather similar in the gel and fluid phases, and quite different from aggregates observed along the gel–fluid transition. Taken together, our data strongly supports that DMPG forms leaky vesicles at both gel and fluid phases.



## 1. Introduction

Dimyristoyl phosphatidylglycerol (DMPG), an anionic saturated phospholipid with two $^{14}$C atoms chains, is the major component of membranes of gram-positive bacteria (Umeyama et al., 2006; Paradis-Bleau et al., 2007). DMPG, mixed with zwitterionic phospholipids, has been used to mimic membranes of bacteria (Chia et al., 2002). DMPG has also been widely used to study the interactions of cationic peptides with lipid bilayers (Biaggi et al., 1997; Prenner et al., 1999; Mozsolits et al., 2001; Turchiello et al., 2002; Amon et al., 2008; Broemstrup and Reuter, 2010), since it is expected that peptides composed of basic amino acids have high affinity for anionic lipid domains in membranes.

In high ionic strength aqueous solutions, DMPG exhibits a highly cooperative gel–fluid phase transition, around 23 °C, similar to the transition of the zwitterionic lipid dimirystoyl phosphatidylcholine (DMPC), which has also two $^{14}$C atoms chains. At low ionic strength, DMPG dispersions exhibit an uncommon thermo-structural behavior. In distilled water, DMPG was reported to form small bilayer "discs or shells" (Epand and Hui, 1986), or vesicles which would fuse in a sponge-like phase below ~31 °C (Gershfeld et al., 1986; Koshinuma et al., 1999). Clearly, the electrostatic repulsion between charged $PG^-$ groups at low ionic strength determines the architecture of DMPG aggregates.

Freshly prepared DMPG dispersions at low ionic strength (Hepes buffer, 0.01 M, pH 7.4) show a wide gel–fluid transition region, between approximately 18 °C ($T_m^{on}$; the onset of the transition) and 35 °C ($T_m^{off}$; the offset of the transition) (see, for instance, Barroso et al., 2010; Alakoskela et al., 2010, and references therein). Along this transition region several heat absorption peaks can be detected (Salonen et al., 1989; Heimburg and Biltonen, 1994; Riske et al., 1997, 1999), and the dispersion shows low turbidity (Heimburg and Biltonen, 1994), high electrical conductivity (Riske et al., 1997; Barroso et al., 2010) and high viscosity (Heimburg and Biltonen, 1994; Schneider et al., 1999; Duarte et al., 2008; Barroso et al., 2010). The structure of DMPG aggregates along this remarkable transition region is still a matter of debate. Because of the high viscosity of DMPG dispersion at the transition region, and on the basis of cryo-transmission and freeze-fracture electron microscopy, it has been proposed that DMPG at low ionic strength forms a lipid network, similar to the so-called sponge phase (Schneider et al., 1999). However, this possibility was ruled out on the basis of experiments that discarded lipid exchange between DMPG structures along the transition region (Lamy-Freund and Riske, 2003;

* Corresponding author. Tel.: +55 11 3091 6829; fax: +54 11 3813 4334.
*E-mail address:* mtlamy@if.usp.br (M. Teresa Lamy).

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Alakoskela and Kinnunen, 2007). It was shown that there is no fusion between DMPG aggregates along temperature variation, hence whatever lipid aggregate is present at a certain temperature (for instance at the gel or fluid lipid phase) it will be present along the whole range of studied temperatures, from 5 to 50 °C, though its form can change.

The purpose of the present work is to examine the structure of DMPG aggregates in the gel and fluid phases (at temperatures below $T_m^{on}$ and above $T_m^{off}$), at low ionic strength. Even outside the transition region there is no consensus about how DMPG is organized in aqueous dispersions. While Schneider et al. (1999) observed vesicular structures for DMPG at the gel and fluid phases, using cryo-transmission and freeze-fracture electron microscopy, Kinoshita et al. (2008), with freeze-etch electron microscopy, detected DMPG vesicles only in the fluid phase.

Three complementary techniques were used here. Electron spin resonance (ESR) of spin labels incorporated into DMPG aggregates in the lipid gel phase clearly indicated that lipids were organized in bilayers and not in micelles or bicelles (bilayer fragments). Quantification of [$^{14}$C]sucrose entrapment by the aggregates showed the presence of highly leaky vesicles, and viscosity data indicated that DMPG aggregates present in the gel and fluid phases were similar. Taken together, our data demonstrate that DMPG in low ionic strength aqueous solutions forms leaky vesicles, both in the gel and fluid phases.

## 2. Materials and methods

### 2.1. Materials

DMPG (1,2-dimyristoyl-sn-glycero-3-[phospho-rac-glycerol] sodium salt), DMPC (1,2-dimyristoyl-sn-glycero-3-[phosphocholine]), 5-PCSL (1-palmitoyl-2-(5-doxyl stearoyl)-sn-glycero-3-phosphocholine), and 16-PCSL (1-palmitoyl-2-(l6-doxyl stearoyl)-sn-glycero-3-phosphocholine) were from Avanti Polar Lipids (Birmingham, AL, USA) and used without further purification. The buffer used throughout was Hepes (4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid), obtained from Sigma–Aldrich (St Louis, MO, USA). Deionized water (Milli-Q Plus, Millipore). [$^{14}$C]sucrose, from Amershan Life Science, has a specific activity of 612 mCi/mmol and a radioactive concentration of 200 Ci/ml. Biodegradable Counting Scintillant Cocktail (BCS) was from Amersham Life Science.

### 2.2. Lipid dispersion preparation

For DMPG and DMPC dispersions, a lipid film was formed from a chloroform solution, dried under a stream of $N_2$ and left under reduced pressure for a minimum of 2 h, to remove all traces of organic solvent. Dispersions were prepared by the addition of Hepes buffer (10 mM adjusted with NaOH to pH 7.4, +2 mM NaCl, final [$Na^+$] = 6 mM) followed by vortexing for 2 min above the lipid gel–fluid transition temperature for DMPC ($T_m$ = 23 °C) or $T_m^{off}$ for DMPG (the end of the gel–fluid transition, $T_m^{off} \sim 35$ °C). As DMPC forms very large multilamellar vesicles, the lipid dispersion was extruded (11 times) through polycarbonate filters (100 nm pores) to yield large unilamellar vesicles (LUV) (Hope et al., 1993). For ESR measurements, spin labels (5-PCSL or 16-PCSL) were added to the lipid chloroform solution at 0.2 mol% relative to the lipid concentration (10 mM), hence no spin–spin interaction was observed. Samples were kept at room temperature and used immediately after preparation. For chromatography, 25 mM DMPG and DMPC were prepared. Viscosity measurements were performed with different DMPG concentrations (see Fig. 7), prepared as described above.

### 2.3. Chromatography

To obtain vesicles with [$^{14}$C]sucrose entrapped inside and outside, (*In/Out*), radioactive sugar was added to the Hepes buffer to a final radioactive count of $40 \times 10^3$ cpm/μl. To maintain [$^{14}$C]sucrose only outside (*Out*), the lipid was first suspended with Hepes buffer as described above (DMPC went through the extrusion process), the sample was kept at the desired temperature (6 or 45 °C) and, after thermal equilibration, the [$^{14}$C]sucrose was added. Samples were kept at the desired temperature before and during the chromatographic procedure, hence not going through the lipid transition temperature.

Both preparations, containing [$^{14}$C]sucrose (*In/Out*) or (*Out*), were passed through a Sephadex G-25 medium column (1.2 cm × 28.6 cm), previously saturated with DMPG or DMPC dispersions, at the temperature of the experiment, and eluted with Hepes buffer. The sample volume was 250 μl and the phospholipid concentration was 25 mM. The chromatographic system was kept at the desired temperature (6 °C or 45 °C). Aliquots of 0.5 ml were collected. For the determination of lipid concentration, the phosphate content of each tube was assayed. Each fraction of the column was radio-assayed by adding an aliquot of 0.4–10 ml of BCS, and counting the [$^{14}$C]sucrose β-radiation activity in a Packard Liquid Scintillation Spectrometer 1600TR.

### 2.4. Determination of trapped [$^{14}$C]sucrose

The percentage of entrapped sucrose was calculated as

$$p = \frac{100R_0}{R} \tag{1}$$

where $R$ is the total amount of radioactivity obtained from all collected fractions, and $R_0$ the total radioactivity from only those that also presented phosphorous content. The experimental apparent internal volume by mol of lipids ($V_{exp}$), in units of l/mol, was expressed as

$$V_{exp} = \frac{p}{100[\text{lipid}]_{eff}} \tag{2}$$

where $[\text{lipid}]_{eff} = f[\text{lipid}]_{ln}$ is defined as an *effective concentration* in mol/l, i.e., the lipid concentration added to the column ($[\text{lipid}]_{ln}$ = 25 mM) corrected by a factor $f$, which is the ratio between the number of moles of lipid collected at $V_o$ (void volume) and the total number of moles of lipid added to the column. Then $(1 - f)$ indicates how much lipid was retained at the top of the column.

Assuming spherical vesicles, with external radius $R$, bilayer thickness $d$ and the same area per lipid head group ($a$) in the internal and external monolayers, the internal volume, $V_T$, can be theoretically calculated:

$$V_T = 1000 \times \frac{aN_A(R-d)^3}{3[R^2 + (R-d)^2]} \tag{3}$$

where $N_A$ is the Avogadro's number, $a$ is expressed in $m^2$ and $R$ and $d$ in m. In an experiment with entrapment of [$^{14}$C]sucrose in the internal volume of the vesicles, and no leak, it would be expected that $V_{exp} = V_T$, $V_{exp}$ measured at the end of the column. Hence, the *trap efficiency at the end of the column*, $E_{ff}$, can be defined as

$$E_{ff} = \frac{V_{exp}}{V_T} \tag{4}$$

### 2.5. Determination of phospholipid concentration

Phospholipid concentration was determined according to Rouser et al. (1970), by the absorbance at 797 nm (Hitachi spectrophotometer). Samples (in general 10–100 μl) from each tube collected from the column, and the sample added to the column,

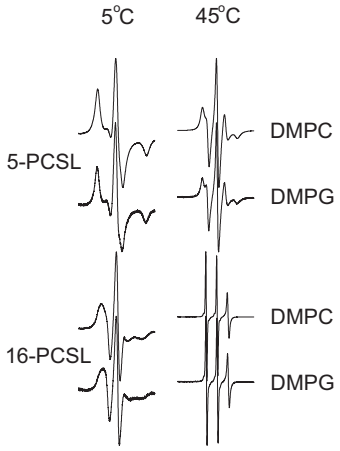


**Fig. 1.** ESR spectra of spin labels (5-PCSL and 16-PCSL) incorporated into 10 mM DMPC and DMPG at 5 °C (left column) and 45 °C (right column). Total spectra width 100 G.

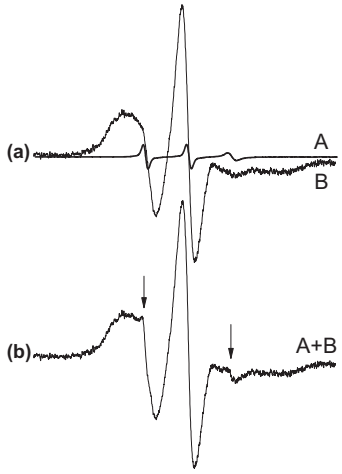


**Fig. 2.** (a) ESR spectra of 16-PCSL incorporated into DMPG at the gel (B, 5 °C) and fluid (A, 35 °C) phases, normalized to relative doubly integrated intensities of 0.99 and 0.1, respectively. (b) The sum of the two signals (A + B), with arrows indicating the features of the spin label incorporated in fluid lipids (A). Total spectra width 100 G.

were assayed, and the amount of phospholipid in each fraction was determined. The concentration of phosphate in each sample was calculated using a standard curve (from 10 to 100 μM of $NaH_2PO_4$) determined with a standard solution of $NaH_2PO_4$ ($1 \times 10^{-3}$ M).

### 2.6. ESR spectroscopy

ESR measurements were performed with a Bruker EMX spectrometer. The sample temperature was controlled within 0.1 °C by a Bruker BVT-2000 variable-temperature device. To ensure thermal equilibrium, before each scan, the sample was left at the desired temperature for at least 10 min. The ESR data were acquired immediately after sample preparation. Field-modulation amplitude of 1 G and microwave power of 10 mW were used.

### 2.7. Viscosity measurements

Viscosities were measured with an Ostwald viscometer (ViscoClock Unit from SCHOTT Instruments, Germany) coupled to a thermostat bath (SCHOTT Instruments, Germany). The temperature was measured with a Fluke 51 K/J thermometer immersed in the bath (precision of 0.1 °C) and allowed to equilibrate for 10 min before data acquisition.

## 3. Results and discussion

### 3.1. ESR of spin labels

ESR spectra of spin labels incorporated into lipid membranes can monitor the bilayer gel–fluid transition, yielding typical, and distinct ESR signals for the gel and fluid phases of the bilayer (see, for instance, McConnell, 1976). Fig. 1 shows the ESR spectra of two phospholipid spin probes, which monitor different depths of a bilayer: 5-PCSL monitors the bilayer micro-environment around the 5th C-atom of the hydrocarbon chain, and 16-PCSL is located at the bilayer core. Differences between ESR spectra of 5- and 16-PCSL are due to the well-known flexibility gradient toward the bilayer core (McConnell and McFarland, 1972). Fig. 1 shows that spectra of 5- or 16-PCSL incorporated into DMPC and DMPG domains are similar, indicating comparable structures for the two lipids, i.e., at 5 °C, ESR spectra are typical of spin probes in gel phase bilayers and at 45 °C ESR spectra indicate fluid membranes. The presence of typical gel bilayer spectra in DMPG low ionic strength dispersions, similar to those of the well-studied DMPC (Fig. 1), is strong evidence that DMPG is mostly organized as bilayers, and not micelles.

The presence of significant amounts of bilayer fragments (bicelles) in DMPG dispersion can also be discarded, as the coexistence of even a small percentage of fluid lipids, corresponding to the bicelle edge, can be clearly detected coexisting with gel bilayers as discussed below. Spin labeled sonicated dispersions of the cationic amphiphile dioctadecyl dimethylammonium bromide (DODAB) (Benatti et al., 2011; Oliveira et al., 2011), which form bicelles (Pansu et al., 1990; Cocquyt et al., 2004) exhibit both gel- and fluid-like spectra. To illustrate the fact that even small amounts of disorganized lipids (fluid) coexisting with gel bilayers can be detected as shown in Fig. 2a the ESR signal of 16-PCSL in gel (5 °C, B) and fluid DMPG (35 °C, A). Spin label relative concentrations are 99:1, gel:fluid, by the calculation of the double integral, which quantifies the amount of resonating spins. In Fig. 2b, where the addition of the two signals is shown (A + B), it can be seen that even 1% of spin labels monitoring a fluid domain (see arrows in Fig. 2b) can be detected in the presence of 99% of labels incorporated in gel bilayers. Even in a very large lipid bicelle, with 50 nm of radius, the fraction of lipids at the edge will be about 1 mol%.

Hence, the observation that spin labels incorporated into DMPG aggregates below 17 °C indicates the presence of gel bilayers only is a strong indication that DMPG, in 10 mM HEPES buffer + 2 mM NaCl, pH 7.4, is mostly organized as bilayers, making vesicles and not micelles or bicelles. A similar conclusion cannot be drawn for DMPG at higher temperatures from ESR spectroscopy. Above the gel–fluid transition of the lipid, it is not possible to distinguish the two ESR components (bilayer and bicelle border) either due to the similarity of the two signals or to the quick exchange of labels between the two domains in the ESR time scale (Benatti et al., 2011; Oliveira et al., 2011).

### 3.2. Entrapment of [$^{14}$C]sucrose

Vesicles are characterized by the presence of an internal water compartment. One method to demonstrate the presence of vesicles in a lipid dispersion is the incorporation of a label in their internal aqueous volume. Radioactive molecules such as sucrose ([$^{14}$C]sucrose) have been widely used to measure the efficiency of encapsulation (Zborowski et al., 1977; Allen and Everest, 1983; Van Hoogevest et al., 1984) and to estimate the internal volume of vesicles (Miyamoto and Stoeckenius, 1971; Carmona-Ribeiro and Chaimovich, 1983). Sucrose is very hydrophilic and does not bind to phospholipids bilayers. Accordingly, we used [$^{14}$C]sucrose to verify

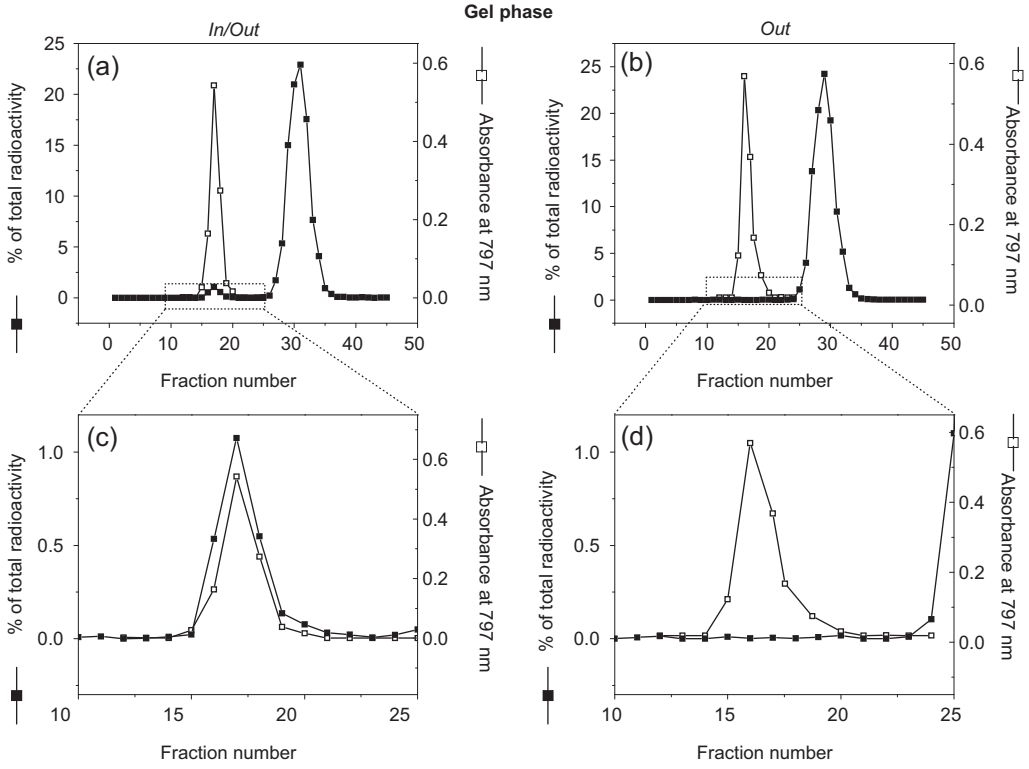


**Fig. 3.** Elution profiles through Sephadex G-25 columns of DMPC vesicles (25 mM) at the gel phase (6 °C), prepared with [$^{14}$C]sucrose (a) *In/Out*, (b) *Out* of the vesicles, (c) and (d) are enlargements of the data in phospholipids containing fractions in (a) and (b), respectively.

whether DMPG aggregates can be demonstrated to have an internal volume in the gel (6 °C) and fluid (45 °C) phases. It was not possible to perform the chromatography along the transition region, as the sample is very viscous, as mentioned in Section 1.

As described in Section 2, vesicles were prepared either (a) in the presence of [$^{14}$C]sucrose, aggregates *In/Out*; or (b) [$^{14}$C]sucrose was added after vesicle preparation, aggregates *Out*. Free and vesicle entrapped [$^{14}$C]sucrose were separated by column filtration (see Section 2). Considering that free [$^{14}$C]sucrose elutes through the column slower than lipid aggregates, free [$^{14}$C]sucrose and vesicle trapped [$^{14}$C]sucrose are expected to be detected in different fractions.

As a control we entrapped [$^{14}$C]sucrose in DMPC dispersions. Figs. 3 and 4 show elution profiles of DMPC vesicles prepared with [$^{14}$C]sucrose *In/Out* (a) and only *Out* (b), in the gel (6 °C, Fig. 3) and fluid (45 °C, Fig. 4) phases of the lipid membrane.

In the gel phase, with sucrose *In/Out* DMPC vesicles, we observed two radioactivity peaks. The first peak (fractions 15–20, Fig. 3a and c), coincided with the phospholipid, strongly suggesting that the [$^{14}$C]sucrose was entrapped in vesicles. The second radioactivity peak (fractions 25–35), corresponds to free [$^{14}$C]sucrose. The percentage of entrapped [$^{14}$C]sucrose, calculated by Eq. (1), was $2.3 \pm 0.1\%$. With [$^{14}$C]sucrose *Out*, the elution profile showed only one radioactivity peak (Fig. 3b and d), in the elution position of free [$^{14}$C]sucrose. Enlarging the scales (Fig. 3d) showed no significant radioactivity in the fractions containing phospholipid in added *Out*, *demonstrating* that no significant amount of sucrose was either incorporated into gel DMPC vesicles or adsorbed from solution.

In the fluid phase of DMPC, with sucrose *In/Out*, the elution profile was similar to that obtained in the gel phase (compare Figs. 3 and 4a and c). Both gel and fluid phases incorporated $2.5 \pm 0.1\%$ [$^{14}$C]sucrose in the phospholipid-containing peak. Interestingly, with [$^{14}$C]sucrose *Out* of the vesicles, we observed a small entrapment of [$^{14}$C]sucrose into DMPC vesicles (Fig. 4b and d, compare with Fig. 3b and d), corresponding to $0.12 \pm 0.01\%$ (see Table 1). This implies that a small influx of [$^{14}$C]sucrose to the internal compartment of DMPC occurs in the time of the experiment.

In experiments where sucrose was added outside LUVs of DMPC and DMPG, vesicles were previously temperature equilibrated and samples were applied to the column immediately after [$^{14}$C]sucrose addition. As permeation is time-dependent, the incubation time of vesicles with sucrose is important in order to obtain incorporation of the probe from outside. These experiments demonstrated that there was no adsorption of [$^{14}$C]sucrose to DMPC vesicles because in the gel phase we found no radioactivity in the vesicle-containing peak, hence a small incorporation of sucrose in the fluid phase of DMPC occurred in the time of the experiment.

Taking the diameter of DMPC vesicles as 100 nm ($R = 50 \times 10^{-9}$ m in Eq. (3), 0.53 and $0.65 \times 10^{-18}$ m$^2$ area per lipid head group ($a$ values in Eq. (3)), and 5.02 and $4.48 \times 10^{-9}$ m bilayer thickness ($d$ values in Eq. (3)) (Marsh, 1990), at gel and fluid phases, respectively, we calculate an internal volume of 2.1 l/mol for the gel phase and 2.7 l/mol for the fluid phase (Eq. (3)). Experimentally determined encapsulated volumes (see Eq. (2)) were $0.9 \pm 0.1$ and $1.0 \pm 0.1$ l/mol, for the gel and fluid phases, respectively, with [$^{14}$C]sucrose *In/Out* the vesicles. Thus, DMPC vesicles were shown to be partially permeable to sucrose, with an entrapment efficiency of ca. $42 \pm 5\%$ in the gel phase, and $37 \pm 4\%$ in the fluid phase (Eq. (4)). Sucrose is more permeable than dextran (a high molecular weight polymer) in multilamellar (MLV) vesicles of DMPC containing 4% egg phosphatidic acid, both below and above $T_m$ (Van Hoogevest et al., 1984). MLVs of DMPC entrap 54% sucrose below $T_m$, while above $T_m$ vesicles only incorporate 30% of sucrose, when compared with dextran. It is not possible to directly compare the entrapment efficiencies measured here (LUVs of DMPC) with those obtained before (MLVs of DMPC; Van Hoogevest et al., 1984). Differences between experimental and theoretical

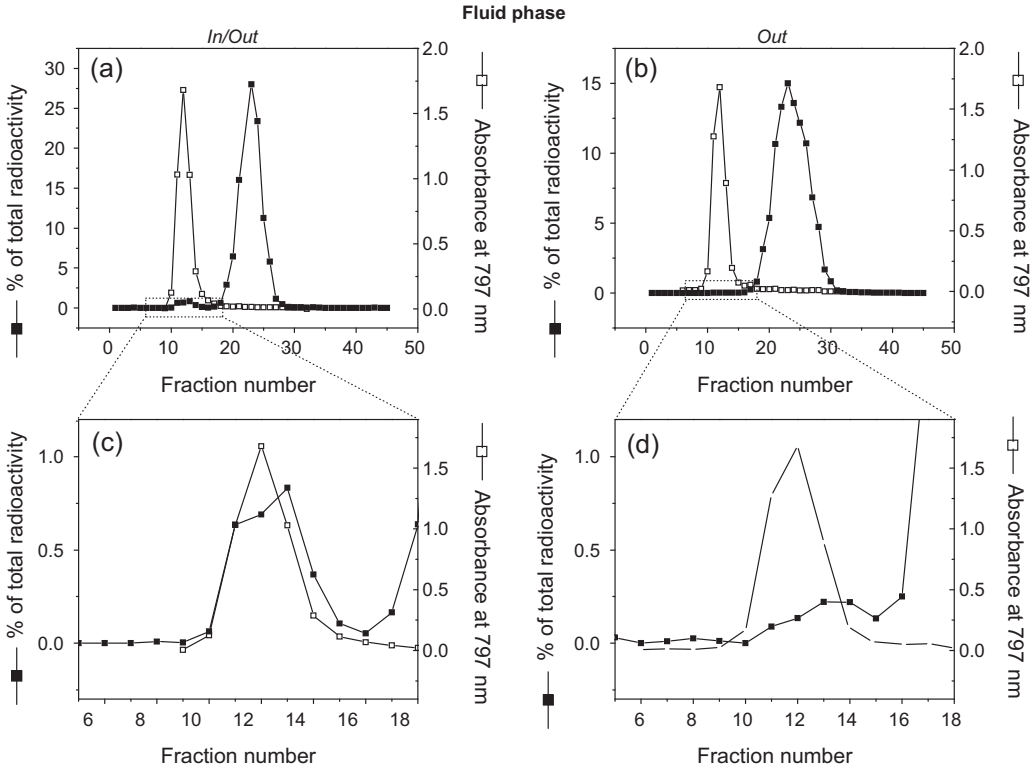


**Fig. 4.** Elution profiles through Sephadex G-25 columns of DMPC vesicles (25 mM) at the fluid phase (45 °C), prepared with sucrose (a) *In/Out* or (b) *Out* the vesicles, (c) and (d) are the amplification of the data in fractions containing phospholipids in (a) and (b), respectively.

entrapment of sucrose in DMPC LUVs, as measured here, are to be expected, due to the known permeability of DMPC vesicles to sucrose, even through multibilayers.

The incorporation experiments described above with DMPC demonstrated the existence of internal volume in a system well characterized as containing vesicles. To monitor the vesicles present in DMPG dispersions, we measured [$^{14}$C]sucrose incorporation in DMPG dispersions.

Fig. 5 shows elution profiles of DMPG aggregates in the gel phase, prepared with [$^{14}$C]sucrose *In/Out* and with [$^{14}$C]sucrose *Out*. In order to demonstrate the reproducibility of our experiments we show the results of two different preparations. No evidence of incorporation of [$^{14}$C]sucrose into DMPG aggregates in the gel phase was found, since no significant radioactivity was detected in the fractions that contained phospholipids: for the four samples the only radioactivity peak detected was that of free [$^{14}$C]sucrose.

For the fluid phase of the lipid, elution profiles of DMPG aggregates are shown in Fig. 6. Differently from the gel phase, we observed a significant, radioactivity peak associated to entrapped [$^{14}$C]sucrose in samples prepared with [$^{14}$C]sucrose *In/Out* vesicles (Fig. 6, profiles on the left side). It is important to note that the recoveries of lipid, after passing DMPG vesicles through the column, are 80% and 90% when the experiment are done below and above $T_m$, respectively. This small amount of lipid stuck at the top of the column was taken into account in Eq. (2), in the calculation of $V_{exp}$.

The percentage of entrapment of [$^{14}$C]sucrose was 0.8 ± 0.1% in one sample (Fig. 6a and c) and 0.5 ± 0.1% in another sample (Fig. 6e and g). In one of the samples prepared with [$^{14}$C]sucrose *Out* (Fig. 6b and d), some [$^{14}$C]sucrose seems to elute with the lipid, which would be an indication of [$^{14}$C]sucrose penetrating DMPG vesicles. However, it is not possible to quantify such entrapment due to the

**Table 1**
Entrapped [$^{14}$C]sucrose ($p$ in Eq. (1)), apparent internal volume ($V_{exp}$ in Eq. (2)), and trap efficiency ($E_{ff}$ in Eq. (4)) of DMPC and DMPG dispersions, in the gel and fluid phases, with sucrose *In*/*Out* and *Out* of lipid aggregates.

| | DMPC | | | DMPG | | |
|---|---|---|---|---|---|---|
| | $p$ (%) | $V_{exp}$ (L/mol) | $E_{ff}$ (%) | $p$ (%) | $V_{exp}$ (l/mol) | $E_{ff}$ (%) |
| Gel phase | | | | | | |
| *In*/*Out* | 2.3 ± 0.1 | 0.9 ± 0.1 | 42 ± 5 | 0 | 0 | 0 |
| | | | | 0 | 0 | 0 |
| *Out* | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | 0 | 0 | 0 |
| Fluid phase | | | | | | |
| *In*/*Out* | 2.5 ± 0.1 | 1.0 ± 0.1 | 37 ± 4 | 0.8 ± 0.1 | 0.35 ± 0.04 | 11 ± 5 |
| | | | | 0.5 ± 0.1 | 0.22 ± 0.04 | 7 ± 3 |
| *Out* | 0.12 ± 0.01 | 0.05 ± 0.01 | 1.9 ± 0.4 | [a] – | – | – |
| | | | | 0 | 0 | 0 |

[a] For one of the DMPG samples, at the lipid fluid phase, it was not possible to calculate reliable values of entrapped sucrose, due to the overlap of the vesicle entrapped sucrose peak and the peak of free sucrose (Fig. 6b and d).

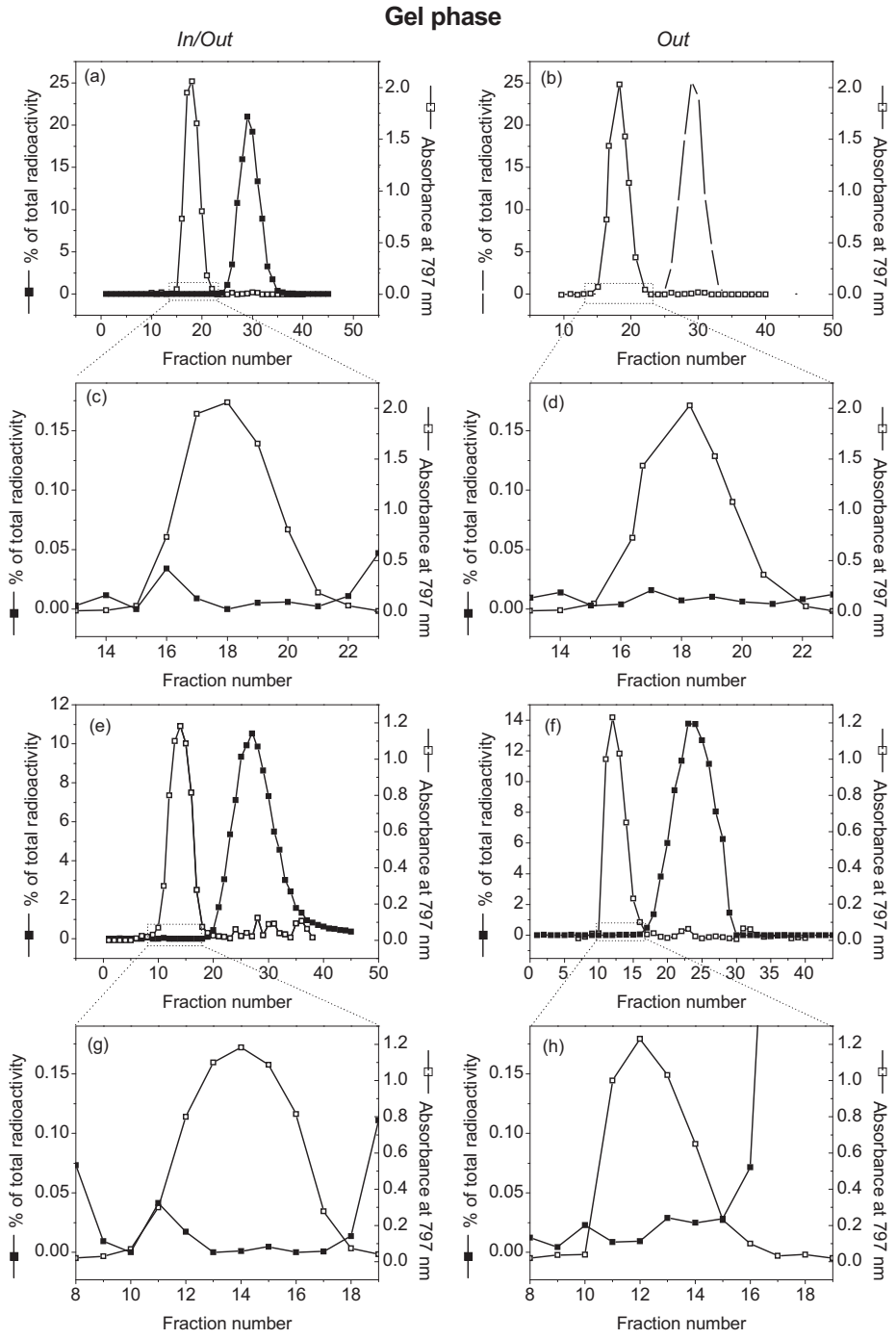


**Fig. 5.** Elution profiles through Sephadex G-25 columns of DMPG aggregates (25 mM) at the gel phase (6 °C), prepared with [$^{14}$C]sucrose *In/Out* ((a) and (e) are two identically prepared experiments), or *Out* ((b) and (f) are two identically prepared experiments) of the vesicles. (c), (d), (g) and (h) are amplifications of the data in fractions containing phospholipids in (a), (b), (e) and (f), respectively.

overlap of the vesicle entrapped sucrose peak and the peak of free sucrose.

Different from DMPC dispersions, DMPG dispersions were not extruded, since no large multilamellar structures are present at low ionic strength (Riske et al., 2001; Fernandez et al., 2008). Considering that the *z*-average radius obtained by dynamic light scattering for DMPG aggregates in the gel and fluid phases, $R \sim 70 \times 10^{-9}$ m (Alakoskela and Kinnunen, 2007), is probably much larger than the number-average radius of the vesicles (see, for instance, Berne and Pecora, 2000), $V_T$ values were calculated assuming $R$ varying from 70 to $50 \times 10^{-9}$ m, and this variation was considered in the calculation of an error in the $V_T$ value. Other parameters used for the calculation of $V_T$ were $0.6 \times 10^{-18}$ m$^2$ and $4.48 \times 10^{-9}$ m, for the area per lipid head group ($a$ value in Eq. (3)) and bilayer thickness ($d$ value in Eq. (3)), respectively (Marsh, 1990). Accordingly, $V_T$ was found to be $3.7 \pm 1.2$ l/mol, compared with $0.35 \pm 0.04$ l/mol (Fig. 6a and c) and $0.22 \pm 0.04$ l/mol (Fig. 6e and g), pointing to the fact that DMPG aggregates are constituted by rather leaky vesicles.

Data discussed above are summarized in Table 1: the percentage of vesicle entrapped [$^{14}$C]sucrose ($p$ in Eq. (1)), the experimental apparent internal volume ($V_{exp}$ in Eq. (2)) and the entrapping

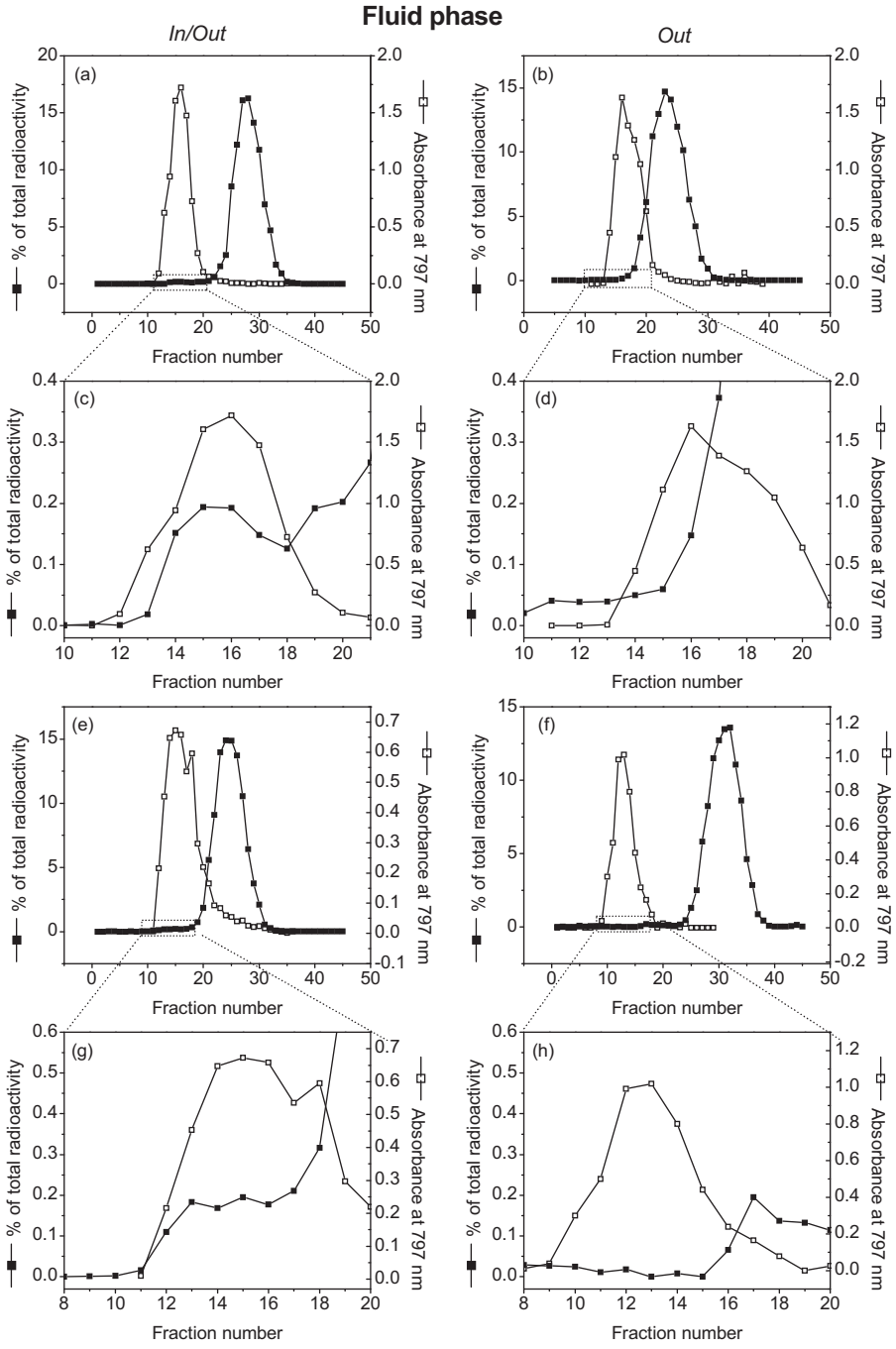


**Fig. 6.** Elution profiles through Sephadex G-25 columns of DMPG aggregates (25 mM) at the fluid phase (45 °C), prepared with [$^{14}$C]sucrose *In/Out* ((a) and (e) are two identically prepared experiments), or *Out* ((b) and (f) are two identically prepared experiments) of the vesicles. (c), (d), (g) and (h) are amplifications of the data in fractions containing phospholipids in (a), (b), (e) and (f), respectively.

efficiency ($E_{ff}$ in Eq. (4)). Clearly DMPG vesicles are partially permeable to sucrose, and this permeability is much greater than that exhibited by DMPC vesicles, since the entrapping efficiency of DMPG vesicles is much lower than that of DMPC vesicles. The presence of the negative charge in DMPG could be responsible for the higher permeability of DMPG LUVs when compared to LUVs of DMPC. Electrostatic repulsion between charged headgroups in the bilayer could increase the distance between monomers and this could lead to an increase in the permeability of the membrane. However, it is important to have in mind that ESR of spin labels incorporated into the bilayer hydrophobic acyl chains indicates similar packing structures for DMPG and DMPC, at gel and fluid phases (Fig. 1).

It is noteworthy that the flow in the column at higher temperature (45 °C), for fluid bilayer vesicles, was around twofold that at lower temperature, 6 °C (gel phase lipids). Therefore, vesicles in the gel phase remained in the column for a longer period of time than those in the fluid phase. Considering that DMPG vesicles are rather permeable to sucrose, this could explain why it was not possible to observe incorporation of sucrose into gel DMPG vesicles. However, for DMPC vesicles, which are also significantly permeable to sucrose, but not as much as those of DMPG, the entrapment

of [$^{14}$C]sucrose for fluid and gel bilayer vesicles, was similar (Table 1).

ESR spectra of spin probes in DMPG aggregates in the gel phase clearly indicated that DMPG is organized as vesicles. In addition, experiments with [$^{14}$C]sucrose entrapment demonstrated the formation of leaky DMPG vesicles. Experiments with spin and fluorescent probes show no fusion of DMPG aggregates over the range of temperature studied here, including the transition region (Riske et al., 1999; Lamy-Freund and Riske, 2003; Alakoskela and Kinnunen, 2007). Furthermore considering that only a small percentage of [$^{14}$C]sucrose was encapsulated by fluid DMPG, we have a strong indication that DMPG leaky vesicles are present in the gel and fluid phases of the lipid. Viscosity experiments discussed below support this hypothesis.

### 3.3. Intrinsic viscosity of DMPG

As mentioned before, one of the unusual characteristics of the transition region of DMPG at low ionic strength is the high viscosity of the dispersion (Heimburg and Biltonen, 1994; Duarte et al., 2008; Barroso et al., 2010). The increase in the viscosity of the dispersion has been associated to structural changes of DMPG aggregates along the transition region (Schneider et al., 1999; Barroso et al., 2010).

The relative viscosity of a dispersion, $\eta_r$, is defined as $\eta_r = \eta/\eta^{buffer}$, where $\eta^{buffer}$ is the pure solvent viscosity and $\eta$ is the dispersion viscosity. At low concentrations, the relative viscosity is a function of the lipid concentration, $c$, and can be expressed as:

$$\eta_r = 1 + [\eta]c + k_1[\eta]^2c^2 + k_2[\eta]^3c^3 + \ldots \quad (5)$$

where $[\eta]$, the intrinsic viscosity, is the main parameter used to obtain information about the size and shape of colloidal particles

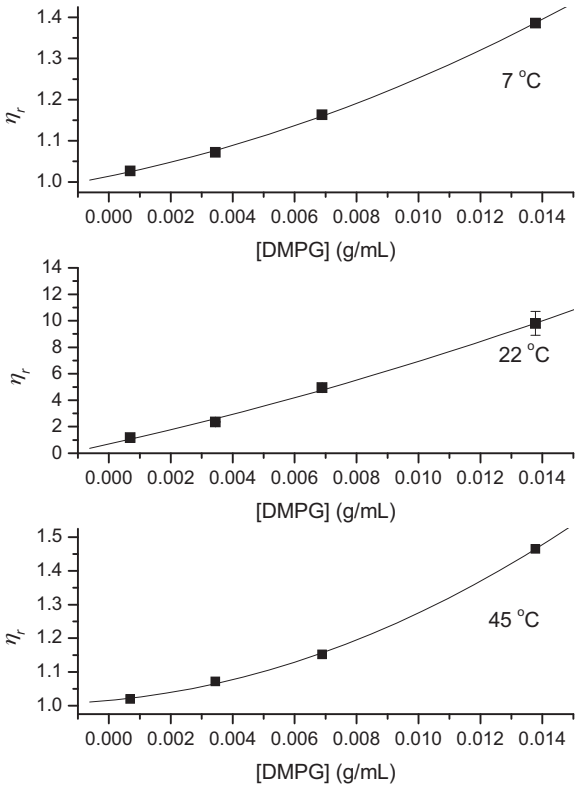


**Fig. 7.** Relative viscosity ($\eta_r = \eta/\eta^{buffer}$) as a function of DMPG concentration, at the gel (7 °C) and fluid (45 °C) phases, and at the transition region (22 °C). Curves are the best fits obtained using Eq. (5), truncated at third term. Data are average values of at least three measurements with different samples. When not shown, the uncertainty (standard deviation) was found to be smaller than the symbol in the graph.

**Table 2**
Intrinsic viscosities of DMPG dispersions, obtained by the best fits of the data in Fig. 5.

| Temperature (°C) | $[\eta]$ (ml/g) |
|---|---|
| 7 | 13 ± 1 |
| 22 | 522 ± 137 |
| 45 | 13 ± 1 |

(see, for instance, Larson, 1999). This is an extension of the fundamental work of Einstein on spheres (Einstein, 1905, 1906).

In Fig. 7, the dependence of the dispersion relative viscosity with DMPG concentration (at low ionic strength) is shown at three temperatures, representative of the gel and fluid phases (7 and 45 °C, respectively) and the transition region (22 °C) (data from Barroso et al., 2010).

The adjustment of second-degree polynomials to the experimental data allowed the calculation of the intrinsic viscosity, $[\eta]$, according to Eq. (5), for the three lipid phases (shown in Table 2).

Identical values obtained for the gel and fluid phases indicate that DMPG aggregates have similar structure at these two conditions, supporting our previous discussion about the presence of DMPG leaky vesicles at both lipid phases.

Though the DMPG transition region is not the focus of the present work, it is interesting to point out that along this anomalous phase of the lipid, the intrinsic viscosity of the dispersion is much higher, consistent with non-spherical shape for the aggregates, the presence of bilayer deformations, including bilayer pores (Riske et al., 2004), or tattered bilayer sheets (Alakoskela and Kinnunen, 2007), and/or the increase on the electrical charge of the DMPG aggregate (Barroso et al., 2010).

## 4. Conclusions

Complementary techniques were used here to show that DMPG, in low ionic strength aqueous dispersion (10 mM Hepes buffer + 2 mM NaCl, pH 7.4), is organized as vesicles, in the gel and fluid phases of the lipid. At low temperatures, ESR of spin labels clearly indicated the presence of gel bilayers only, showing that DMPG is organized as vesicles for temperatures below the gel–fluid transition of the lipid. The analysis of the ESR signal excludes the presence of a significant amount of bicelles, where a gel bilayer coexists with a fluid bilayer edge. Therefore, considering the presence of DMPG vesicles, the absence of [$^{14}$C]sucrose entrapped by gel phase DMPG vesicles revealed that the vesicles are rather leaky. For higher temperatures, the small percentage of entrapped [$^{14}$C]sucrose by fluid DMPG aggregates, and the similarity between intrinsic viscosity values measured for DMPG at gel and fluid phases, strongly indicated that DMPG was also organized as leaky vesicles.

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